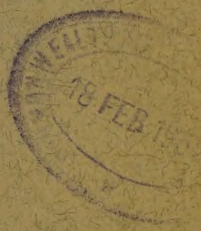
Volume 26 OCTOBER, 1951 Number 1

IOWA STATE COLLEGE JOURNAL OF SCIENCE

A Quarterly of Research



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PUBLISHED BY
THE IOWA STATE COLLEGE PRESS
PRESS BUILDING
AMES, IOWA

IOWA STATE COLLEGE
JOURNAL OF SCIENCE

Published October, January, April, and July

Published under the joint auspices of the graduate faculty of Iowa State College and the local chapter of Sigma Xi. The administrative board for the academic year 1950–51 includes:

Manuscripts should be submitted to R. E. Buchanan, 24 Curtiss Hall, Iowa State College, Ames, Iowa.

All matter pertaining to subscriptions, remittance, etc., should be addressed to the Iowa State College Press, Press Building, Ames, Iowa. Subscriptions are as follows: Annual: $6.00 (in Canada $6.50; other foreign countries $7.00); single copies: $2.00 (except No. 2 in each volume, beginning with Vol. XXV, $3.00).

Entered as second-class matter January 16, 1935, at the post office at Ames, Iowa, under the act of March 3, 1879.

EFFECT OF BENZENE AND SIX SELECTED SALICYLATES ON THE DEVELOPMENT OF IMMUNITY IN *TRYPANOSOMA LEWISI* INFECTION AND ON VARIOUS ASPECTS OF THE BLOOD PICTURE[1]

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Received March 10, 1951

Brown (4) noted that laboratory rats which were infected with *Trypanosoma lewisi* showed two distinct immunological reactions to the parasite. One reaction checked the multiplication of the flagellates; the other destroyed them. Taliaferro (12) supplied serological evidence for two separate plasma reaction-products: a reproduction-inhibiting antibody, "ablastin," and a trypanocidal antibody, "trypanolysin." The presence of the two antibodies in the blood of infected rats appeared to be responsible for the development of immunity to the trypanosome.

Recent work by Becker and Gallagher (2), Becker and Lysenko (3), and Saul and Becker (10) revealed that adequate daily dosage with sodium salicylate interfered with the production or action of ablastin in *T. lewisi*-infected rats. Results with sodium salicylate suggested the desirability of testing the effect of other salicylates on the immune reaction of rats infected with the same trypanosome. It seemed probable that treatment with benzene might yield interesting results, since it is known to inhibit the production of lysins [Hektoen (7)]. The purpose of the present investigation was to determine the effect of six salicylates and benzene on the course of *T. lewisi* infection and on various aspects of the blood picture.

MATERIALS AND METHODS

The host animals were male, inbred, "Wistar A" rats, two to three months old. The rats were louse-free and exhibited no *Bartonella muris* infection. All animals, control and test, were infected with Becker's strain of *T. lewisi*. On the zero day of the experiment each rat received intraperitoneally 1.0 to 2.0 milliliters of blood diluted with normal saline, containing between 100,000 and 2,000,000 trypanosomes. The approximate number injected was estimated with the hemacytometer.

The substances listed in Table 1 were administered daily to groups of test rats, beginning with the zero day and continuing throughout the

[1] Condensed from doctoral thesis number 1035, two copies of which are on file in the Iowa State College library. The work was supported in part by a grant from the Industrial Science Research Institute, Iowa State College, and was done under the direction of Dr. Elery R. Becker.

course of the infection. Control rats received daily equivalent amounts of the carrier substance. Both test and control feedings were introduced directly into the stomach of the rat by means of a No. 8 soft rubber catheter attached to a 2.0-milliliter syringe.

Blood samples were obtained from the tip of the rat's tail on the zero day of the experiment, on the third day of the infection, and on alternate days thereafter. Hemoglobin readings and total leucocyte and trypanosome counts were made on the fresh blood. Blood smears were

TABLE 1
SUBSTANCES FED TO TEST RATS

Test Substance	Daily Dose	Solvent—1.0 to 2.0 ml. %
Methyl salicylate	1.0 to 2.0 ml.	12% Gum arabic (aqueous)
Acetylsalicylic acid	45 to 98 mg.	35% Ethyl alcohol or 12% Gum arabic (aqueous)
Para-aminosalicylic acid	50 to 100 mg.	Distilled water
Phenyl salicylate	45 mg. per 100 g.	Distilled water
Sodium salicylate	45 mg. per 100 g.	Distilled water
Salicylic acid	45 mg. per 100 g.	Distilled water
Thiophene-free benzene	0.1 to 0.2 ml. per 100 g.	12% Gum arabic (aqueous)
Ethyl alcohol (35%)*	2.0 ml.	.....

* Included as a test substance since it was used as a solvent for acetylsalicylic acid, and there was some question concerning its effect on the usual course of *T. lewisi*-infection.

stained in Wright's and were used in obtaining differential leucocyte counts and percentages of division forms of trypanosomes.

Hemoglobin determinations in grams per 100 cubic centimeter of blood were made with a Spencer Hb-meter. Total leucocyte counts were obtained by the standard hemacytometer method after dilution with Tuerk's solution. The total number of trypanosomes per milliliter of blood was determined by the standard hemacytometer method for counting erythrocytes, the blood being diluted with Hayem's solution and twenty squares being counted.

Differential leucocyte counts usually were based on the examination of 200 white cells on each stained smear. When, however, the leucocyte count was greatly reduced, as in benzene treatment, the differential count was sometimes based on examination of only 100 cells. Monocytes and lymphocytes were classified as "mononuclear leucocytes."

A trypanosome showing a dividing or divided blepharoplast was considered a reproducing parasite and was designated a "division form." The percentage of division forms usually was obtained from each stained

smear by examination of 500 to 1,000 trypanosomes. When, however, parasites became very scarce, it was sometimes necessary to base this percentage on examination of only 100 trypanosomes. Parasites usually continued to vary in size for a short time after division forms could no longer be found. The approximate time at which the adult stage of the infection was reached was estimated by determining the time at which a trypanosome population ceased to vary in size.

RESULTS

COURSE OF THE INFECTION

THE AVERAGE INFECTION IN UNTREATED RATS

The average control infection, based on data for twenty-seven untreated, infected rats, became established in the peripheral blood by the third day after trypanosomes were inoculated. In the blood, the parasites underwent active multiplication which resulted in a peak trypanosome population of 122,800 per cubic millimeter of blood on the sixth day. Reproduction, as shown by the percentage of division forms, ceased by the end of the fifth day, indicating that ablastin was exerting its anti-multiplicative action by that time. All parasites attained adult size by the eighth day. Within two to four days after the peak, a sharp decrease in the number of adult trypanosomes occurred. This was followed by further decreases, usually more gradual, until the infection was terminated on the seventeenth day.

Blood samples takes at the time of the first sharp decrease in trypanosomes revealed two interesting phenomena: first, a large percentage of parasites occurring in the stained smears had aparently undergone lysis; second, most of the flagellates in the fresh blood were agglutinated in masses which were so firmly held together that they often appeared intact even in stained blood smears. Agglutination was general among parasites of control rats from the fifth day of the infection until all parasites disappeared from the blood.

THE INFECTION IN RATS TREATED WITH ETHYL ALCOHOL OR PARA-AMINOSALICYLIC ACID

Neither ethyl alcohol nor para-aminosalicylic acid in the amounts used affected the course of the typical *T. lewisi* infection as outlined above. Data presented in Table 2 illustrate the similarity between test and control infections.

THE INFECTION IN RATS TREATED WIH METHYL SALICYLATE, ACETYLSALICYLIC ACID, PHENYL SALICYLATE, SODIUM SALICYLATE, SALICYLIC ACID, OR BENZENE PLUS SODIUM SALICYLATE

These substances in the dosages used tended to alter the course of the typical *T. lewisi* infection in much the same way. The parasite in treated rats remained in the multiplicative phase throughout the course of the infection. Figure 1 illustrates the extremely high and characteristic reproductive rate. Size variation among the individual parasites is great, and the most common types of division forms are

TABLE 2

COURSE OF THE INFECTION IN RATS TREATED WITH ETHYL ALCOHOL, PARA-AMINOSALICYLIC ACID, OR BENZENE AND IN THEIR NONTREATED CONTROLS

	Control							Test							
Treatment	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Last Seen Day	Tryps. All Adult Day	Inf. Term Day	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Last Seen Day	Tryps. All Adult Day	Rat Died Day	Inf. Term Day
Ethyl alcohol	3I64	3	125.0	9	9	11	29	3I67	3	152.5	5	5	7	.....	19
	3I65	3	182.5	5	5	11	15	3I68	7	107.5	9	9	11	.....	21
	3I66	3	272.5	7	7	9	19	3I69	5	150.0	9	9	11	.....	25
Para-amino-salicylic acid	4J17	7	20.0	10	7	10	.....	4J18	3	5.0	3	.....	5	.....	7
	.....	.....	.....	.....	.....	.....	.....	4J19	3	92.5	5	5	7	.....	10
Benzene	8P28	5	20.0	5	5	7	15	8P30	3	212.5	5	5	7	30(S)	.....
	8P29	3	32.5	5	3	5	9	8P31	3	152.5	5	5	7	30(S)	.....
								8P32	3	227.5	5	3	9	28	.....
								8P33	3	190.0	5	5	9	28	.....
	8Q36	3	130.0	5	7	9	17	8Q38	3	117.5	5	3	7	.....	15
	8Q37	3	217.5	5	5	7	15	8Q39	3	227.5	5	5	7	.....	19
	.....	.....	.....	.....	.....	.....	.....	8Q40	3	225.0	5	3	5	.....	31
	.....	.....	.....	.....	.....	.....	.....	8Q41	3	220.0	9	3	7	.....	19
	8R48	3	125.0	7	3	7	17	8R52	3	182.5	5	5	7	.....	21
	8R49	3	147.5	5	3	7	13	8R53	3	292.6	5	5	7	.....	19
	8R50	3	185.0	5	3	7	17	8R54	3	180.0	7	5	9	.....	50
	8R51	3	157.5	5	5	7	11	.....	.....	.....	.....	.....	.....	.....	.....

S—Sacrificed

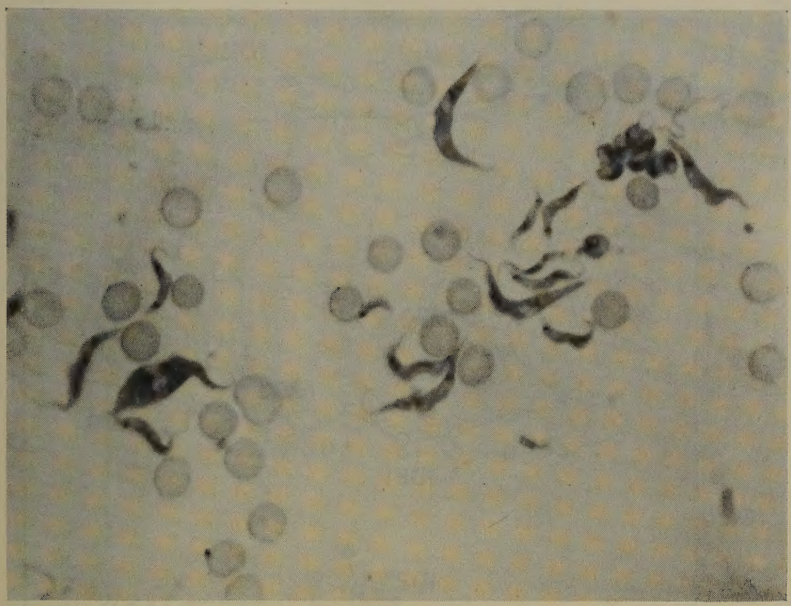

FIG. 1.—Multiplying trypanosomes in blood of test rat number 1D25, treated with methyl salicylate, thirteenth day of the infection (x900).

present. Near the upper right of Fig. 1 is a rosette of eight small trypanosomes. Such rosettes were indicative of extremely active multiplication on the part of the parasites and were rarely seen in control infections after the third or fifth day. Near the lower left is a large trypanosome whose blepharoplast has already divided preparatory to longitudinal binary fission of the cytoplasm. Figures 2 and 3 show the persistence of division forms in test rats long after reproduction has ceased in control rats.

Unless death of the host intervened, continued multiplication of the trypanosomes usually resulted in a flagellate population considerably larger than that of control rats and delayed the time of the peak. Consequently the infection tended to be lengthened, not only in the few rats which survived but also in those which succumbed. Data shown in Figs. 2 and 3 and Table 3 illustrate these points for treated rats as compared with their untreated controls. The control infection shown in Fig. 2 was most typical of untreated rats, while that shown in Fig. 3 was the densest encountered in the entire investigation. It will be noted in Fig. 3 that although the peak control trypanosome population exceeded the peak test flagellate population in one instance, the reduction in the total parasite count which followed the peak was much sharper in the control, and less time was required for the control rat to eliminate the infection.

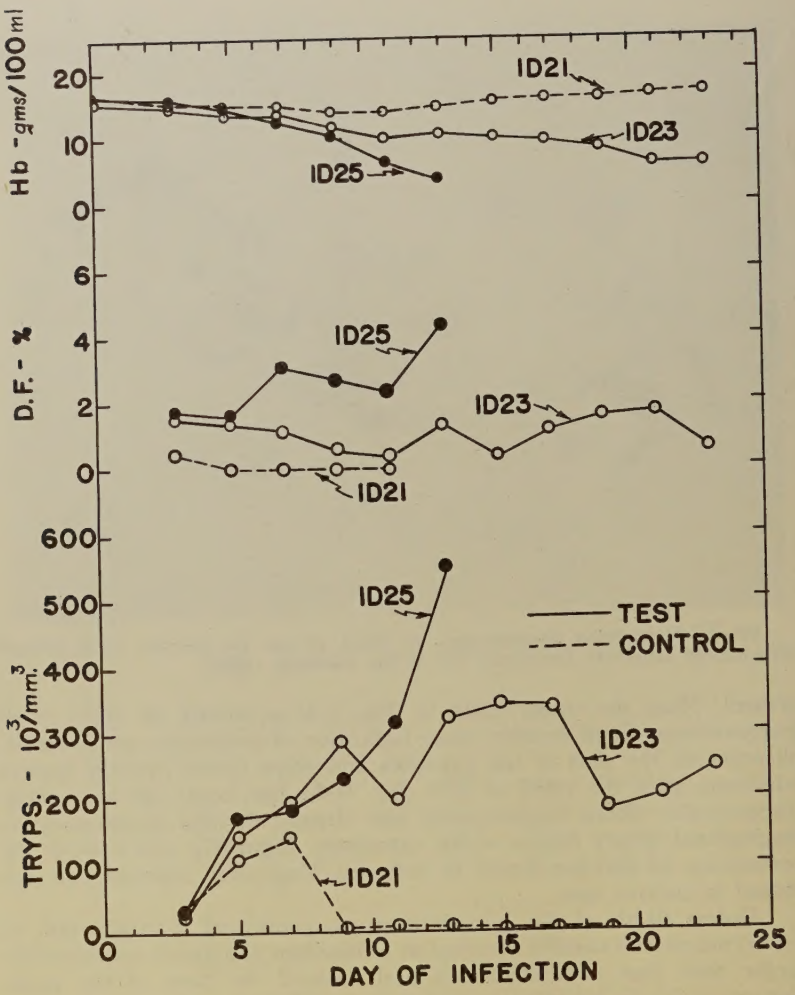

FIG. 2.—Course of the trypanosome infection, division forms, and hemoglobin concentration of the blood for two test rats, treated with methyl salicylate, and for one control.

Table 3 shows that the majority of the salicylate-treated rats died. The infection in these cases followed two general lines, both of a pathogenic nature. Some animals died at the height of the infection without showing definite evidence of host resistance to an ever-increasing parasite population. Infections in these rats resembled pathogenic trypanosome infections of the continuous type (see Fig. 2, test rat number

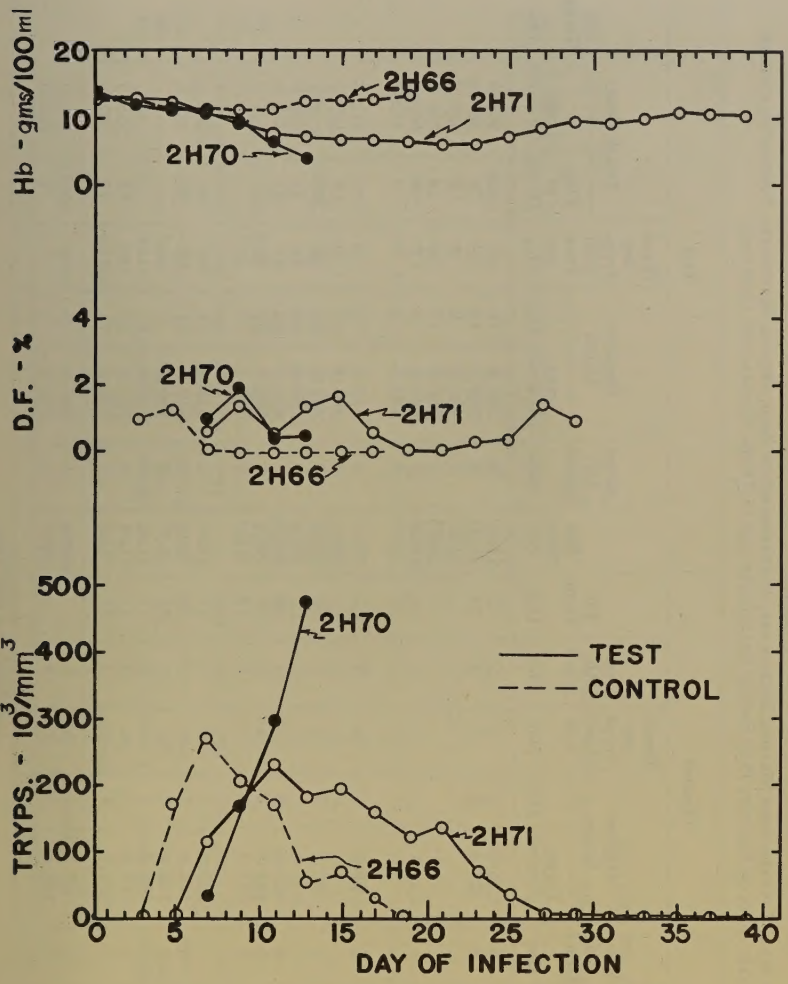

FIG. 3.—Course of the trypanosome infection, division forms, and hemoglobin concentration of the blood for two test rats, treated with acetylsalicylic acid, and for one control.

1D25; Fig. 3, test rat number 2H70). Other animals developed periodic host resistance which was manifested in temporary sharp decreases in the parasite population. However, continued reproductive activity by the remaining trypanosomes, often at a higher rate than was shown when no resistance was evident, served to bring about subsequent increases in the parasite population and to cause the infection to take a course

TABLE 3

COURSE OF THE INFECTION IN RATS TREATED WITH METHYL SALICYLATE, ACETYLSALICYLIC ACID, PHENYL SALICYLATE, SODIUM SALICYLATE, SALICYLIC ACID, OR BENZENE PLUS SODIUM SALICYLATE AND IN THEIR NONTREATED CONTROLS

	Control							Test							
Treatment	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Last Seen Day	Tryps. All Adult Day	Inf. Term Day	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Still Present Day	Death Data Day of Death	Death Data Tryps. * $10^3/mm^3$	Inf. Term Day
Methyl salicylate	1D20	3	35.0	3	5	7	23	1D22	3	505.0	11	11	12	505.0	.....
	1D21	3	124.5	7	3	5	19	1D23	3	342.5	15	23	24	250.0	.....
	.....	.....	.....	.....	.....	.....	.....	1D24	3	420.0	8	8	8	420.0	.....
	.....	.....	.....	.....	.....	.....	.....	1D25	3	550.0	13	13	14	550.0	.....
	.....	.....	.....	.....	.....	.....	.....	1D26	3	147.5	5	5	6	147.5	.....
	.....	.....	.....	.....	.....	.....	.....	1D27	3	322.5	5	9	9	152.5	.....
Acetylsalicylic acid	2F11	3	50.0	7	5	7	.....	2F12	3	312.5	10	10	12	312.5	.....
	.....	.....	.....	.....	.....	.....	.....	2F13	3	187.5	7	8	8	187.5	.....
	2G14	3	87.5	7	5	7	30	2G15	3	305.0	16	30	30(S)	3.8	.....
	.....	.....	.....	.....	.....	.....	.....	2G16	5	1240.0	14	14	15	1240.0	.....
	2H64	3	125.0	9	9	11	29	2H70	7	475.0	13	13	14	475.0	.....
	2H65	3	182.5	5	5	11	15	2H71	3	232.5	11	29†	.....	.....	39
	2H66	3	272.5	7	7	9	19	2H72	3	202.5	7	21†	.....	.....	37
Phenyl salicylate	5K73	3	205.0	7	5	9	17	5K79	3	422.5	11	17	19	265.0	.....
	5K74	5	115.0	7	5	7	15	5K80	3	330.0	11	19	21	222.5	.....
	5K75	3	130.0	5	5	9	15	5K81	3	190.0	9	19†	.....	.....	23
	5L82	3	105.0	5	5	7	17	5L88	7	167.5	13	19†	.....	.....	24
	5L83	3	115.0	5	3	7	17	5L89	3	355.0	13	13	15	355.0	.....
	5L84	3	110.0	5	5	7	17	5L90	3	597.5	11	11	12	597.5	.....
Sodium salicylate	6M91	3	87.5	5	3	5	.....	6M96	3	285.0	9	9	10(S)	285.0	.....
	6M92	3	130.0	7	7	9	.....	6M97	3	230.0	7	7	9	230.0	.....

(continued on next page)

TABLE 3 (*continued*)

Treatment	Control							Test							
	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Last Seen Day	Tryps. All Adult Day	Inf. Term Day	Rat Number	Blood Inf. Establ. Day	Highest Count Tryps. $10^3/mm^3$	Highest Count Day	Dividing Tryps. Still Present Day	Death Data Day of Death	Death Data Tryps. * $10^3/mm^3$	Inf. Term Day
Salicylic Acid	7N73	3	205.0	7	5	9	17	7N76	3	245.0	11	19‡	25(S)	Trace	.....
	7N74	5	115.0	7	5	7	15	7N77	3	417.5	11	11	12	417.5	.....
	7N75	3	130.0	5	5	9	15	7N78	3	525.0	13	15	15	432.5	.....
	7O82	3	105.0	5	5	7	17	7O85	3	370.0	13	13	13	370.0	.....
	7O83	3	115.0	5	3	7	17	7O87	3	582.5	11	11	12	582.5	.....
	7O84	3	110.0	5	5	7	17	.....	.....	.....	.....	.....	.....	.....	.....
Benzene plus Sodium Salicylate	9S43	3	170.0	5	3	7	13	9S44	3	287.5	9	19	20	117.5	.....
	.....	.....	.....	.....	.....	.....	.....	9S45	3	337.5	7	9	9	337.5	.....
	.....	.....	.....	.....	.....	.....	.....	9S46	3	222.5	5	9	10	212.5	.....
	9T91	3	87.5	5	3	5	.....	9S47	3	270.0	5	7	8	195.0	.....
	9T92	3	130.0	7	7	9	.....	9T94	3	37.5	9	9	10(S)	37.5	.....
								9T95	3	252.5	7	9	10(S)	245.0	.....

S—Sacrificed.
* Trypanosomes showed much variation in size to end of infection
† Last count prior to death
‡ Trypanosomes still variable four days later.

resembling that of pathogenic infections of the relapsing type. (See Fig. 2, test rat number 1D23.)

Agglutinated masses of parasites, such as were regularly noted in the blood of control rats after the fifth day, were never observed in rats treated with methyl salicylate, acetylsalicylic acid, phenyl salicylate, soduim salicylate, salicylic acid, or benzene plus sodium salicylate. However, lysed remnants of trypanosomes appeared coincidentally with periods of decline in the total parasite count, indicating that trypanolysin was present.

THE INFECTION IN RATS TREATED WITH BENZENE

The usual course of the *T. lewisi* infection was altered in some respects by the daily administration of benzene alone. Differences in treated and untreated infections are illustrated by the data shown in Table 2. It will be seen that, whereas the peak of the infection was reached at approximately the same time in test and control animals, the average parasite count at the peak was greater for the test rats (202,500 as compared with 126,900). Following the peak, the total number of trypanosomes was reduced in both test and control animals, but the reduction was more gradual in the test rats. Consequently, the average length of the infection was extended from 14.3 days in untreated rats to 26.4 days in the benzene-treated. This was a significant difference at the 1 per cent level.

Examination of stained blood showed that the daily administration of benzene had no effect on either the time at which parasites ceased to reproduce or the time at which the adult stage of the infection was reached. Also, benzene did not interfere with agglutination of adult trypanosomes. Treated and nontreated rats alike showed agglutinated masses of parasites from the fifth day until late in the infection. Some lysis also was noted.

BLOOD-STUDY RESULTS

HEMOGLOBIN

All rats, control and test, suffered reductions in hemoglobin during the course of the infection. The extent of the reductions seemed to be roughly proportional to the density of the flagellate population except in test rats which received benzene alone. The usual pattern included: (1) a gradual reduction as the parasites increased in number, the lowest hemoglobin value being recorded at the time of or shortly after the peak of the infection; and (2) a gradual increase toward normal as the infection subsided. Occasionally, the initial reduction in hemoglobin was preceded by a slight temporary increase. Figures 2 and 3 show the relationship which existed between the density of the trypanosome population and the extent of the reduction in hemoglobin. They also illustrate the recovery picture for the two controls and for one test rat. It will be noted that the hemoglobin concentration of test rat number 2H71 shown in Fig. 3 was approaching normal by the end of the infection

although acetylsalicylic acid had been administered for forty days. Figure 4 further bears out the assumption that the hemoglobin reduction was a direct result of the infection. Invasion of the blood was not accomplished in the test rat shown in Fig. 4 until the seventh day after intraperitoneal inoculation of trypanosomes. Phenyl salicylate had been administered daily since the zero day. Nevertheless, the first appreciable reduction in hemoglobin occurred on the ninth day, two days after parasites appeared in the blood. Furthermore, recovery of normal hemo-

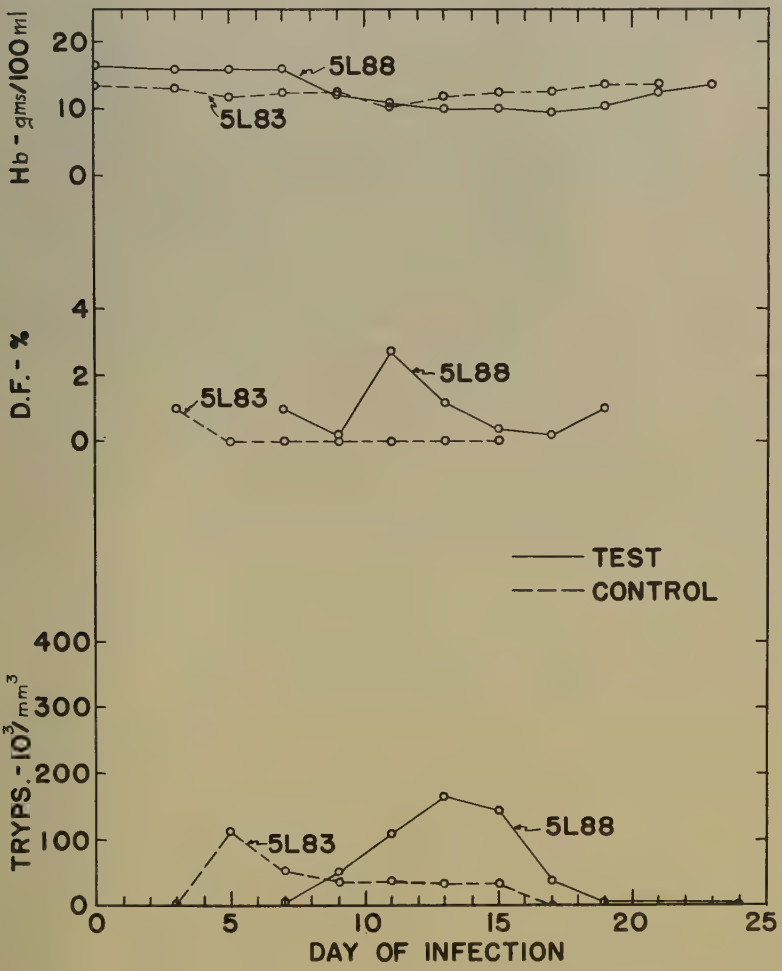

FIG. 4.—Course of the trypanosome infection, division forms, and hemoglobin concentration of the blood for one test rat, treated with phenyl salicylate, and for one control.

globin concentration was almost complete with the disappearance of the flagellates, although phenyl salicylate had been administered for twenty-five days.

Rats which received benzene, alone, showed a fluctuating hemoglobin value, characterized by periodic decreases and increases. Sometimes a slight initial increase preceded the first decrease. The lowest hemoglobin determinations recorded for benzene-treated rats closely approximated those recorded for nontreated controls. In the case of benzene treatment, however, the lowest hemoglobin concentrations were not correlated with the peak of the infection but were recorded as early as the fifth day in some cases or as late as the twenty-seventh day in others. Hemoglobin concentration returned to normal soon after the discontinuance of benzene treatment at the termination of the infection. The slight effect of benzene on hemoglobin was not evident, if present, when benzene was given in conjunction with sodium salicylate. Animals receiving both substances coincidentally developed intense infections, and hemoglobin concentration was reduced proportionally.

TOTAL LEUCOCYTE COUNT

Changes in the total leucocyte count seemed to be correlated with the trypanosome infection in untreated rats and in treated rats except those which received benzene or benzene plus sodium salicylate. The density of the parasite population apparently exerted no influence on the magnitude of the effect. Hence, control and test animals showed essentially the same leucocyte picture. The average normal leucocyte count closely approximated 10,000 cells. This number usually was reduced 2,000 to 4,000 cells between the time trypanosomes were injected and the time the blood was invaded. The initial decrease was followed by a gradual increase in leucocytes which brought the count up to normal by the peak of the infection. The increase in white cells continued, resulting in a leucocytosis which raised the average count 8,000 to 9,000 cells above normal and which lasted until the infection terminated. Occasionally, the initial decrease either did not occur or was over by the time three-day counts were made.

Benzene or benzene plus sodium salicylate initiated a gradual or fluctuating decrease in leucocytes which was usually followed by a fluctuating increase toward normal. Only one treated rat of these two groups of animals regained the initial number of white cells while benzene was being administered. The lowest leucocyte counts for rats treated with benzene alone ranged from 300 to 2,000 cells and were recorded between the tenth and the thirtieth day of treatment. Leucocytes of rats treated with benzene plus sodium salicylate were reduced to 1,500 to 3,600 by the sixth or tenth day of treatment. Many of the animals died before these counts could be confirmed as the lowest which might have been obtained.

DIFFERENTIAL LEUCOCYTE COUNT

The average differential leucocyte count for all rats prior to treatment or inoculation with trypanosomes was as follows: mononuclears,

72.8 per cent; neutrophils, 26.6 per cent; eosinophils, 0.4 per cent; basophils, 0.2 per cent. As the infection progressed all rats, control as well as test, developed a slight basophilia which elevated the average incidence of basophils to 0.5 per cent. The effect was the same regardless of treatment or size of the trypanosome population.

Control rats and rats treated with ethyl alcohol, para-aminosalicylic acid, methyl salicylate, acetylsalicylic acid, phenyl salicylate, sodium salicylate, or salicylic acid showed one effect on other white cells; those treated with benzene or benzene plus sodium salicylate showed another. The average mononuclear percentage for the former groups of animals was increased to 76 per cent, and the average incidence of neutrophils was decreased accordingly. Again the effect was the same in treated or nontreated rats, in heavy or light trypanosome infections. The eosinophil percentage was unaffected.

In contrast to the slight increase in mononuclear leucocytes noted above, animals treated with benzene or benzene plus sodium salicylate suffered a marked decrease in these cells. The average mononuclear percentage of 72.8 per cent was reduced to 50.3 per cent with benzene and to 54.3 per cent with benzene plus sodium salicylate. The average percentage of neutrophils increased from the normal of 26.6 per cent to 49.3 per cent with benzene treatment and to 45.1 per cent with benzene plus sodium salicylate. The normal incidence of eosinophils was reduced from 0.4 per cent to 0.07 per cent with benzene treatment, to 0.1 per cent with benzene plus sodium salicylate.

Evidence that benzene, either alone or in conjunction with sodium salicylate, attacked the mononuclear leucocytes directly was provided by the degenerative changes which appeared within many of these cells after five days of treatment. Numerous clear, vacuole-like areas formed within the cytoplasm, serving to distort the normal shape of the cell and to give it a moth-eaten appearance.

DISCUSSION

Untreated rats normally developed three types of host resistance to *T. lewisi*: ablastic, which brought parasite reproduction to an end; trypanolytic, which killed many of the adult trypanosomes by lysis; and agglutinating, which caused adult parasites to cohere in groups. Ablastin and trypanolysin have been thoroughly discussed in the literature [see Taliaferro (13)]. In view of reference by various authors to a true agglutinin which caused trypanosomes to cohere to one another in the blood of infected rats [Laveran and Mesnil (9)] and which foretold the disappearance of the parasites from the circulation [Francis (6)], probably by filtration in the liver and spleen [Augustine (1); Taylor and Becker (14)], it appears that the importance of agglutination in the development of immunity to *T. lewisi* is not sufficiently appreciated at the present time. The writer believes that the agglutinin noted in the blood of control rats of the current investigation played an important role, in conjunction with trypanolysin, in bringing infections to an end. This belief is supported by the fact that agglutinated masses of

parasites first appeared at the time of the sharp decrease in the trypanosome population which followed the peak of the infection. After its first appearance, agglutination continued throughout the period of decline in the flagellate population.

It is well known that *T. lewisi* is normally nonpathogenic for laboratory rats. Under treatment with the salicylates employed in the investigation (para-aminosalicylic acid excluded, but benzene plus sodium salicylate included), however, *T. lewisi* frequently became a pathogen. It is believed that pathogenicity can be attributed to failure of ablastin and the agglutinin under salicylate treatment. Failure of ablastin was indicated by the prolonged reproductive period of the parasites and by the accelerated rate of multiplication during periods of declining trypanosome count. Failure of the agglutinin was indicated by the complete absence of agglutinated masses of flagellates in the blood.

The only one of the usual types of host resistance which appeared to be active in the salicylate-treated rats under consideration was trypanolysin. The presence of trypanolysin was indicated by the appearance in the blod of lysed remnants of trypanosomes at times of temporary or permanent decrease in the parasite population. It seems plausible that, in the absence of other forms of host resistance, an excessive amount of trypanolysin might be required to achieve reductions in trypanosome populations which were being continually enlarged by apparently uninhibited reproductive activity. The extra time needed for trypanolysin titre to reach an effective concentration may have contributed to the dense trypanosome populations and the delay in reaching the peaks characteristic of salicylate-treated rats.

Furthermore, the fate of such rats may have hinged on the proficiency of their response to the infection by the elaboration of large amounts of trypanolysin. If this were true, continuous pathogenic infections could be attributed to failure of the rat to produce trypanolysin early enough or in sufficient amount to stay the upward trend in the parasite population. Relapsing pathogenic infections could be attributed to a trypanolysin titre of sufficient strength to cause periodic sharp reductions in the parasite population but of insufficient strength to prevent subsequent increases in the total number of trypanosomes. Nonpathogenic infections in salicylate-treated rats could be attributed to early production of an efficient trypanolysin which held the peak flagellate count lower than that encountered in pathogenic infections and which killed the parasites faster than they could be replaced by reproduction.

Trypanolysin, and not ablastin or agglutinin, seemed to be the antibody affected by administration of benzene alone. Cessation of parasite reproduction at the expected time indicated that ablastin was present. Agglomerations of trypanosomes, occurring coincidentally with the decline in parasites following the peak, indicated that agglutinin also was present. Apparently benzene acted to decrease the titre of trypanolysin and not to prevent its formation. The appearance of lysed

remnants of trypanosomes during the decline in the parasite population after the peak indicated that trypanolysin was present. The slightly higher than usual trypanosome count at the peak, the more gradual reduction in the total number of parasites which followed, and the lengthened infection all indicated that trypanolysin was of subnormal titre.

Since sodium salicylate apparently interferes with ablastin and agglutinin and benzene with trypanolysin, it might be expected that administration of both substances would result in *T. lewisi* infections of the continuous pathogenic type. Such infections, however, did not develop under the conditions of the current investigation. Infections in rats receiving both benzene and sodium salicylate closely paralleled those in rats receiving salicylate alone, and any depressive effect which benzene may have had on trypanolysin was inapparent.

The data demonstrated that the trypanosome infection ordinarily was accompanied by certain changes in the blood picture; i.e. reduction in hemoglobin, leucopenia followed by leucocytosis, slight increase in mononuclears and basophils, slight decrease in neutrophils, and no change in eosinophils [see Duca (5)]. Only the reduction in hemoglobin seemed to be influenced by the density of the parasite population. Hence, those rats which developed severe infections as a direct result of salicylate therapy also suffered more severe reductions in hemoglobin.

Benzene is known to attack the blood and blood-forming organs directly, the effect on erythrocytes being relatively slight, that on leucocytes being much more severe. According to Latta and Davies (8) benzene, in the dosage used in the current investigation, alternately stimulates and depresses the rat's erythrocytogenic centers. This might account for the periodic fluctuations in hemoglobin which were characteristic of benzene-treated animals. The fluctuations were never great and so were inapparent in rats treated with benzene plus sodium salicylate where the larger effect of the trypanosome population on hemoglobin was noted.

The leucocyte picture, both total and differential, was altered much more by benzene therapy than by the trypanosome infection. Hence, rats receiving benzene alone or benzene plus sodium salicylate showed essentially the same leucocytic changes. Reports of other authors, [Selling (11) and Latta and Davies (8)] and results of the current investigation indicate that the characteristic effect of benzene may be attributed to destruction of the circulating leucocytes and to degeneration followed by regeneration within the leucocyte-forming organs. The data suggested that mononuclear leucocytes and eosinophils were most affected by benzene.

CONCLUSIONS

1. Untreated, *T. lewisi*-infected rats developed agglutinin in addition to ablastin and trypanolysin. Agglutinin appeared to be partly responsible for clearing the blood of adult trypanosomes.

2. Neither the course of the infection nor the immune reaction of the host was affected by the daily administration of ethyl alcohol or para-aminosalicylic acid.

3. In rats treated with methyl salicylate, acetylsalicylic acid, phenyl salicylate, sodium salicylate, salicylic acid, or benzene plus sodium salicylate, the usual course of the infection was altered in the following respects: (a) The reproductive phase of the parasites was prolonged. (b) Continued multiplicative activity of the trypanosomes resulted in dense parasite populations. (c) The peak of the infection was delayed. (d) The infection often took a pathogenic course, either continuous or relapsing. (e) The infection tended to be prolonged.

4. The above deviations from the usual course of the infection were attributed to: (a) Failure of ablastin and agglutinin in the presence of salicylate. (b) Variation among the individual rats in the amount of trypanolysin produced and in the time of its first appearance in the blood.

5. In rats treated with benzene alone, the usual course of the infection was altered in the following respects: (a) The trypanosome population at the peak was larger. (b) The parasites were removed from the blood less efficiently. (c) The duration was definitely prolonged.

6. The above deviations from the usual course of the infection were attributed to a benzene-induced decrease in the titre of trypanolysin.

7. Nontreated, *T. lewisi*-infected rats and infected rats which received ethyl alcohol, para-aminosalicylic acid, methyl salicylate, acetylsalicylic acid, phenyl salicylate, sodium salicylate, or salicylic acid showed the following deviations from the normal blood picture: (a) Reduction in hemoglobin roughly proportional to the density of the flagellate population. (b) Initial leucopenia, followed by leucocytosis persisting until the infection was terminated. (c) Slight increase in mononuclear leucocytes, accompanied by a comparable slight decrease in neutrophils. (d) Slight basophilia.

8. The above deviations from the normal blood picture were attributed directly to the trypanosome infection.

9. The trypanosome-induced basophilia was regularly noted in rats treated with benzene or benzene plus sodium salicylate.

10. Treatment with benzene, alone, caused small fluctuating increases and decreases in hemoglobin which may have been due to alternate stimulation and depression of the erythrocytogenic centers.

11. In rats treated with benzene plus sodium salicylate, salicylate indirectly affected hemoglobin concentration by favoring a large trypanosome population, but direct effects of benzene were not evident.

12. Benzene, either alone or in conjunction with sodium salicylate, destroyed many leucocytes of the circulating blood, selecting mononuclears and eosinophils for destruction.

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THE MAGNETIC SUSCEPTIBILITY OF VITAMIN B_{12a}

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Received March 13, 1951

In an earlier paper (1) we reported that vitamin B_{12} is diamagnetic. We have now measured the magnetic susceptibility of a specimen of vitamin B_{12a} of high purity. This is of interest for it now appears that B_{12a} [formed by catalytic hydrogenation (2) and subsequent oxygenation of B_{12}] is identical with B_{12b} [formed by fermentation (3)] and that B_{12a} and B_{12} are the hydroxide and cyanide, respectively, of the same cobalt-organic complex (4).

Like vitamin B_{12}, vitamin B_{12a} is diamagnetic and thus contains either trivalent cobalt or bivalent cobalt bearing molecular oxygen as discussed previously (1); as would be expected, the replacement of cyanide by hydroxyl does not change the magnetic character of the material.

EXPERIMENTAL WORK

MATERIALS

The vitamin B_{12a} used was prepared in the Squibb Institute for Medical Research. The method of preparation was that of Kaczka, Wolf, and Folkers (2); the material was twice crystallized from acetone water. Countercurrent distribution in butanol-ammonium sulfate and propanol-ammonium sulfate systems indicated an inhomogeneity of less than 3 per cent. Measurement of the absorption spectrum gave values of $E^{1\ pct.}_{1\ cm.}$ of 163 at 351 millimicrons, 142 at 274 millimicrons, and 59 at 525 millimicrons, after correction for the moisture content (11.51 per cent). An analysis of this material gave: carbon, 55.75; hydrogen, 6.69; nitrogen, 13.10; cobalt, 4.75; phosphorus, 2.52. All calculations were made on the anhydrous basis.

A solution of nickel chloride for standardization was prepared from crystalline nickel chloride hexahydrate containing 10 p.p.m. or less each of iron, cobalt, and copper (J. T. Baker Chemical Co., Phillipsburg, N. J.), and standardized electrolytically. The volume susceptibility of this solution, which contained 30.01 per cent nickel chloride, was 13.128 c.g.s.u./cm^3 (5).

[1] The authors wish to express their appreciation to Miss M. Moore, Mr. F. Russo-Alesi, Dr. N. Coy and Mr. J. Alicino, all of E. R. Squibb & Sons, New Brunswick, N. J., for the preparation, countercurrent distribution studies, determination of the absorption spectrum, and elementary analysis, respectively; the authors are indebted to the Institute for Atomic Research of Iowa State College for making available the magnetic susceptibility apparatus.

APPARATUS

The measurements were made by the Gouy method using the same apparatus described in our earlier paper but with the following modifications. A 0.01 ohm resistor was placed in series with the winding of the magnet and the voltage drop over this resistor measured with a Leeds and Northrup Type K-2 potentiometer; in this way the current energizing the magnet was measured. Aside from a large line voltage regulator preceding the rectifier power supply, no attempt was made to maintain the current constant at any given value; an accurate current measurement and reference to the calibration curve are completely satisfactory for obtaining accurate and reproducible results with the apparatus. The tube bearing the material being measured was surrounded with a vertical glass jacket through which water from a nearby constant temperature bath was passed. The measurements were thus made at $20° \pm 0.05°$.

The tube used for this measurement was a glass tube, 3.8 millimeters in internal diameter and extending 20 centimeters on each side of the septum, that is, to well outside the field of the magnet. Measurements on the empty tube showed that it was magnetically symmetrical and free of any paramagnetic material.

PROCEDURE

The solid vitamin B_{12a} was well packed into the tube by tapping the tube one hundred times after each small addition of material. Sufficient time was allowed for the tube and contents to come to the temperature of the water in the jacket and measurements were made at various field strengths obtained by successively varying the current in steps of about 1 ampere.

A calibration curve for the tube was prepared by plotting changes in weight, measured for the standard nickel chloride solution, against coil current. Calibration data were taken at more than twenty-five currents throughout the range of the power supply.

RESULTS

Vitamin B_{12a} gained weight slightly at low field strengths (currents up to 3 amperes) and then steadily lost weight as the field was progressively increased (currents up to 10 amperes), shown in Fig. 1. It is apparent that a small amount of a ferromagnetic impurity was present in the sample and that B_{12a} is diamagnetic, for the decrease in weight was proportional to the square of the field strength for values greater than that necessary to first saturate the ferromagnetic impurity. The effect of the ferromagnetic impurity was eliminated by drawing a line through the origin parallel to one through the observed values over the range in which the decrease was linear with the square of the field strength, Fig. 1. The decrease in weight from $H^2 = 0$ to $H^2 = 100 \times 10^6$ was then taken from this corrected line and used to calculate the magnetic susceptibility.

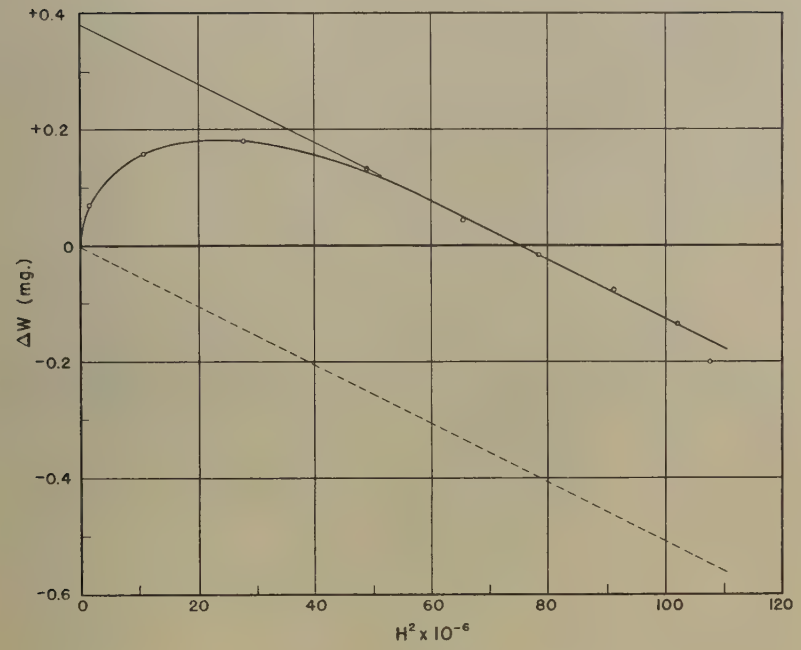

FIG. 1.—Change in weight of vitamin B_{12a} in magnetic field.

The values of H^2 were calculated from the changes in weight for the standard nickel chloride solution (taken from the calibration curve at those currents used in the B_{12a} measurements), the volume susceptibility of the solution, the cross sectional area of the solution and the acceleration of gravity, by the relation

$$H^2 = \frac{2\,g\,\Delta\,w}{A\,(k_{NiCl_2} - k_{air})}$$

The volume susceptibility was calculated by the relation

$$k_{B_{12a}} - k_{air} = \frac{\Delta\,w_{B_{12a}}}{\Delta\,w_{NiCl_2}}\,(k_{NiCl_2} - k_{air})$$

The data obtained are:

Wt. B_{12a} in tube: 1.3218 g. (18 cm. length)

Wt. $NiCl_2$ solution in tube: 2.7727 g. (18 cm. length)

Density $NiCl_2$ solution: 1.345 g./cm^3

Volume of tube filled: 2.062 cm^3

Bed density B_{12a}: 0.6412 g./cm^3

$\Delta w_{B_{12a}}$ corrected $= -0.51$ mg. at $H^2 = 100 \times 10^6$

$\Delta w_{NiCl_2} = 76.57$ mg. at $H^2 = 100 \times 10^6$

$$k_{B_{12a}}, \text{ corrected } -0.029 \times 10^{-6} = \frac{(-0.51)\ (13.1 \times 10^{-6})}{76.57}$$

$$= -0.087 \times 10^{-6}$$

$k_{B_{12a}}$, corrected $= 0.058 \times 10^{-6}$ c.g.s.u./cm^3

(Strictly this is the volume susceptibility of the column)

Taking as the density of B_{12a} 1.34 g./cm^3, the actual volume occupied by the B_{12a} is 0.478 cm^3 per cm^3 of column. There is thus 0.522 cm^3 of air per cm^3 of column. Using the relation

$$k_{column} = (\text{Vol. frac. air})\ k_{air} + (\text{Vol. frac. } B_{12a})\, k_{B_{12a}}$$

gives

$$k_{B_{12a}} = -0.153 \times 10^{-6} \text{ c.g.s.u./cm}^3$$

and

$$\chi_{B_{12a}} = -0.114 \times 10^{-6} \text{ c.g.s.u./g.}$$

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NOTES ON THE DIGGER-BEE, *ANTHOPHORA OCCIDENTALIS*, AND ITS INQUILINES[1]

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Received March 20, 1951

The interrelationships of the associations of various species of the genus *Anthophora* and their inquilines have been reported by numerous investigators. However, studies pertaining to *Anthophora occidentalis* Cresson and its associate species are more limited. Among these are the observations of Mickel (5) who listed ten associate species reared from cells of *A. occidentalis*. *Melecta californica miranda* (Fox) was first reported as this species' associate by Cockerell in 1899 according to Linsley (3). Hicks (2) reared *Hornia neomexicana* (Cockerell) from cells of *Anthophora occidentalis*. More recently, the bionomics of the genus *Hornia* was presented by Linsley and MacSwain (4).

This study concerns *Anthophora occidentalis* Cresson[2] as the host species and certain inquilines which develop in its cells. These inquilines, representing three orders, are: *Melecta californica miranda* (Fox)[2], *Anthrax daphne* (O.–S.)[3], *Hornia neomexicana* (Cockerell)[4], and *Nemognatha bicolor* Lec.[5] All materials were obtained and observations made at Lubbock, Texas.

OBSERVATIONS AND RESULTS

During early winter of 1949–50 more than 400 mud cells (Fig. 1) of *Anthophora occidentalis* were collected and taken to the laboratory for observation. These cells were gathered from tunnels located in vertical banks of an abandoned ground water reservoir. They contain the pollen cache as well as the young or associate of the host bee. The cell is approximately 2.3 centimeters long and 1.3 centimeters wide with a general oval appearance except for the flat capped end. This end of the cell opens directly into the main tunnel or a short branch leading to it. A cluster of cells frequently has a common opening but with an arrangement which permits each individual to pass directly to the exterior.

[1] A thesis submitted to the Graduate Faculty of Texas Technological College in partial fulfillment of the requirements for the degree of Master of Arts. The study was under the direction of Dr. R. W. Strandtmann.
[2] Determined by K. V. Krombein.
[3] Determined by W. W. Wirth.
[4] Determined by G. B. Vogt.
[5] Determined by R. C. Froeschner.

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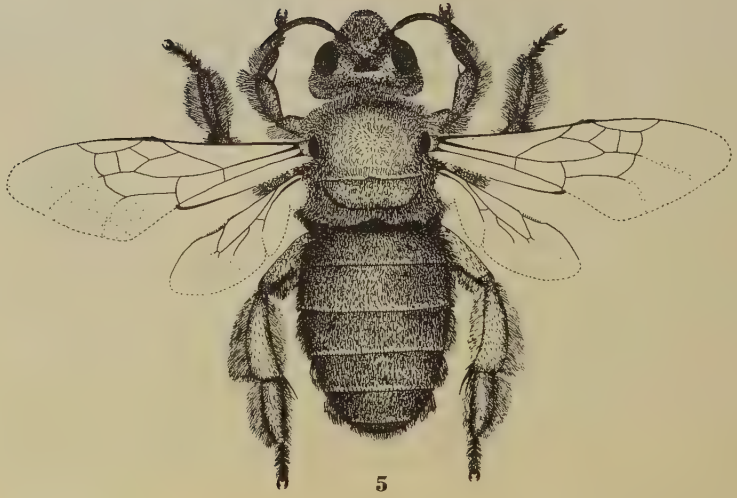

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FIG. 1.—Mud cell of *Anthophora occidentalis*, 1.7x.
FIG. 2.—Mature larva of *A. occidentalis*, 2.1x.
FIG. 3.—Pupa of *A. occidentalis*, 3.8x.
FIG. 4.—Egg of *A. occidentalis*, 8.4x.
FIG. 5.—Adult *A. occidentalis*, 3.8x.

ANTHOPHORA OCCIDENTALIS, THE HOST BEE

The immature stages of the larva are spent in the mud cell provided for it by the female. This species hibernates as a mature larva (Fig. 2). The length of pupation, based on thirty-four observations, is between twenty and twenty-one days, the shortest being seventeen and the longest twenty-three. Pupae (Fig. 3) become abundant in the field about the middle of May. The adult (Fig. 5) escapes by gnawing through the capped end of the cell. This bee is proterandrous, with males appearing in the latter part of May and females following five to seven days later.

The adult bees become abundant by the latter part of June and daily activity about the colony sites is greatest during the cool morning hours. At midday both sexes retire to shade, resting in the openings of the tunnels or hanging by their mandibles and legs from small twigs and leaves of nearby trees and shrubs.

During this season the nesting female utilized old tunnels mined in former years. It takes approximately three days to complete one cell. She spends the first day cleaning out debris in the old tunnel, digging a concavity at the end of the main tunnel or one of its branches, and molding the cell and the chimney at the opening of the tunnel. This concavity holds the cell, and the earth excavated from it is used in building both the cell and the chimney. During this time the bee leaves her work periodically. Presumably, she goes to get water because throughout the periods of daily activity these bees can be seen taking water from nearby ponds. Furthermore, one can actually see the earth being moistened as she molds the chimney. On the second day she places pollen in the cell, and on the third she partially fills the cell with a pungent smelling liquid, deposits her egg, and then seals the cell. The egg (Fig. 4) is approximately 3.8 millimeters long and floats on this liquid mass. Water loss is reduced by a thin waxy membrane that lines the interior of the cell. The liquid is absorbed by the cell wall if this membrane is broken.

MELECTA CALIFORNICA MIRANDA, A BEE INQUILINE

The mature larva (Fig. 7) hibernates in the mud cell within a parchment-like covering. The average length of pupation, based on three observations, is approximately twenty days.

The female (Fig. 9) places her eggs in the cell of the host while it is being made. The egg (Fig. 6) is about 1.5 millimeters long. In contrast to the host which never places but one egg in a cell, *M. californica miranda* may place two or three eggs in each.

HORNIA NEOMEXICANA, A BEETLE INQUILINE

This associate hibernates as a mature larva (Fig. 12) in the mud cell of the host. The pupation period, based on a single observation, was twenty-two days. In the field, pupae (Fig. 13) become abundant about the middle of May with the adults appearing in the latter part of the

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FIG. 6.—Egg of *Melecta californica miranda*, 13x.
FIG. 7.—Mature larva of *M. californica miranda*, 4.5x.
FIG. 8.—Pupa of *M. californica miranda*, 3.6x.
FIG. 9.—Adult *M. californica miranda*, 3.6x.
FIG. 10.—Pupa of *Anthrax daphne*, 3.0x.
FIG. 11.—Adult *A. daphne*, 3.0x.

month and after the middle of June no adult larvae were found.

The adults (Fig. 14) are sluggish with vestigial elytra, a specialization to be associated possibly with a parasitic mode of existence. Because of their sluggish nature, they spend their adult life close to the place where they emerge. The males creep over the embankment and find their way to the cells containing the famales which remain in the cells in which they developed. Mating and ovipositing occurs here. A female produces between 600 and 700 eggs (Fig. 16) and each is approximately 1.3 millimeters in length.

In about twenty days the eggs begin to hatch and at first the small primary larvae (Fig. 15) are white but turn brown in about two hours. In contrast to the adults, the larvae are quite active. They are easily excited by moving objects which cause them to run wildly about with their heads raised and mandibles spread wide. A unique characteristic of the larvae of this species, as well as of other members of the genus, is the presence of an anal gland secretion from which a web is spun. This web is used for lowering and anchoring themselves.

At midday the larvae retreat to shady spots on the embankment and it is probable that at this time when the bees are resting in the tunnels they become infested with these larvae. As many as twenty of them have been observed attached by their mandibles to the hairs of the host bee and, in all probability, are transported in this way to the cells by the bees. Larvae were also found on *Melecta californica miranda* but not on *Anthrax daphne.*

NEMOGNATHA BICOLOR LEC., A BEETLE INQUILINE

This associate hibernates and pupates in the mud cell within a brown, parchment-like capsule (Fig. 17) which is approximately 1.4 centimeters in length. It is of interest that only three of these beetles emerged from the cells under laboratory observation. On June 29, adults (Fig. 18) were observed taking nectar from the wavy-leaved thistle on which *Anthophora occidentalis* was also feeding. Since both of these species feed at times on the same plant, it is suggested that this is a possible means of infestation.

ANTHRAX DAPHNE, A FLY INQUILINE

The mature larva hibernates in the mud cell. The length of the pupation period, based on seven observations, is between twenty-three and twenty-four days. At maturity the pupa (Fig. 10) bores through the cell wall by means of the strong teeth at the anterior end of the pupal skin. The pupa is quite active and by its ability to move freely forces itself out of the cell; the adult then emerges from the pupal skin through a dorsal, anterior slit.

The adult (Fig. 11) is the first member of this association to emerge in the laboratory as well as in the field. The first insects of this species were observed in the field on May 7. Throughout the day both sexes fly lazily before an embankment where the females of *Anthophora occidentalis* engage in their nesting activities.

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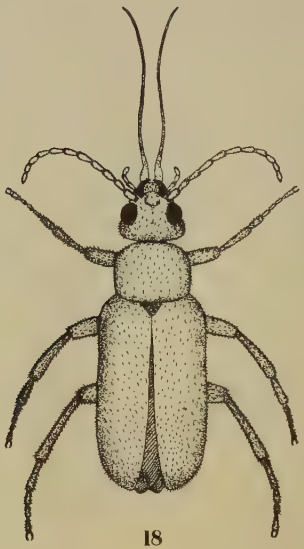

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FIG. 12.—Mature larva of *Hornia neomexicana*, 3.3x.
FIG. 13.—Pupa of *H. neomexicana*, 3.3x.
FIG. 14.—Adult *H. neomexicana*, 3.3x.
FIG. 15.—Primary larva of *H. neomexicana*, 10x.
FIG. 16.—Egg of *H. neomexicana*, 10x.
FIG. 17.—Hibernating case of *Nemognatha bicolor*, 3.3x.
FIG. 18.—Adult *N. bicolor*, 3.3x.

During the time of oviposition, the female flies slowly back and forth before a colony site. When she approaches the tunnel which she has selected for her eggs, she hovers in mid-air about 2 inches before its opening. Then, with darting movement toward the tunnel and with simultaneous curving of the abdomen so that the tip is directed forward, she throws the egg inside. However, it should be emphasized that she is not highly selective in placing her eggs, as tunnels are used indiscriminantly as well as cracks in the embankment. The eggs are about 0.5 millimeter long and the number deposited on each hover varies widely.

From 436 sound mud cells observed under laboratory conditions, 281 insects of five species were reared. Within an infested cell only one insect matures because the successful development of the inquiline results in the destruction of the host. Data relative to the numbers and sex ratios of laboratory reared insects is presented in Table 1; whereas, the relative emergence times of these insects are summarized in Fig. 19.

DISCUSSION

In the study reported here four associate species were reared from Texas-collected cells of *Anthophora occidentalis* which is in contrast to the ten reported by Mickel (5) from cells of the same species collected in Colorado. Of the 85 insects of *A. occidentalis* reared by Mickel (5) he indicated 55 males and 30 females. In the present study, based on 156 reared individuals, 87 were females and 69 were males. Frison (1) found the male-female ratio of *Anthophora abrupta* Say to be about 2:1. It is to be stressed that for these observations only sound cells were used, thereby reducing the possibility of rearing insects other than those which entered the cells during their construction. It was also noted that the life cycle of each member of this association appears to be completed in one year.

Statistical analysis of the emergence data summarized in Fig. 19 indicates that there is no significant difference between the mean emer-

TABLE 1

Specific and Sex Ratios of Laboratory Reared Insects

	Number Reared	Per Cent of Total	Males	Per Cent Males	Females	Per Cent Females
Anthophora occidentalis	156	55.5	69	44.2	87	55.8
Melecta californica miranda	21	7.4	9	42.9	12	57.1
Anthrax daphne	41	14.6	18	48.6	19	51.4
Hornia neomexicana	60	21.4	27	45.0	33	55.0
Nemognatha bicolor	3	1.1				
Total	281					

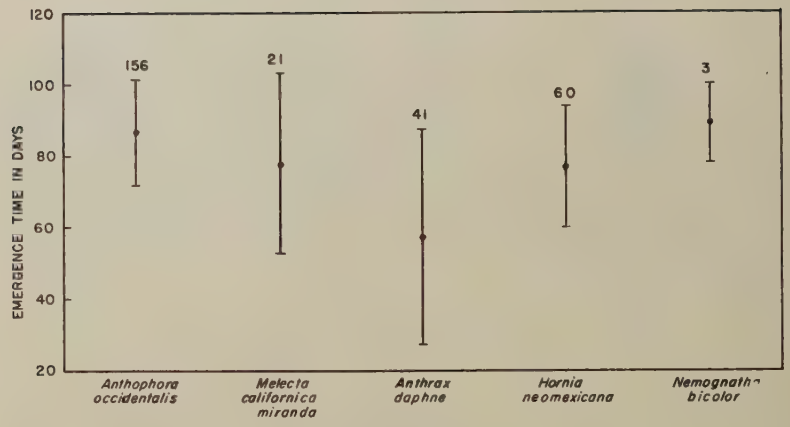

FIG. 19.—Emergence times expressed as means with standard deviations. Number of insects indicated above each mean.

gence times of *A. occidentalis* and that of *Melecta californica miranda*. However, there is a significant difference between the emergence time of the host and that of *Anthrax daphne* and *Hornia neomexicana*. These are significant at P = .01. Analysis was not made of the data for *Nemognatha bicolor* because only three insects were reared. Analysis of variance was used in this study (6).

SUMMARY

Observations are presented dealing with association relationships of *Anthophora occidentalis* Cresson and four associate species, *Melecta californica miranda* (Fox), *Anthrax daphne* (O.–S.), *Hornia neomexicana* (Cockerell), and *Nemognatha bicolor* Lec. Each of these species has been studied in the field as well as the laboratory. Data on behavioral activities of each species, relative emergence times, sex ratios, and degrees of infestation are also reported.

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SOME BACTERIOLOGICAL ASPECTS OF SPOILAGE OF SELF-SERVICE MEATS[1]

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Received April 13, 1951

Self-service merchandising of packaged meat is a relatively new but a rapidly expanding form of retail marketing. Considerable research and study have been given to the problems of packaging and, while many problems have been solved, a larger number have not been worked out successfully. Foremost among these is the increased possibility of microbiological spoilages resulting from mishandling.

Meat serves as an excellent medium for the growth of many types of microorganisms. Workers are not in agreement regarding the source of these contaminants. Reith (30) concluded that bacteria are present in the muscular tissue of apparently normal animals. On the other hand, the data cited by Burn (3,4) and by Jensen and Hess (15) indicate that bacterial invasion is agonal and postmortem rather than antemortem.

Whatever their source, usually a small residual bacterial population can be found in freshly killed tissue and, considering the numbers of organisms on the skin of the animal at the time of slaughter, the surprising thing is that contamination of the deeper flesh is not greater. For example, Jensen and Hess (15) indicated the presence of from 10^5 to 1.5×10^9 aerobes and from 10^4 to 2×10^9 anaerobes on 2 square inches of neck skin of unwashed hogs at the site where the jugular vein is stuck.

According to Moran (22) and Mallmann *et al.* (21) most of the problems associated with the spoilage of meat are surface problems and among causal factors bacteria are of primary importance. Moran and Smith (23) reported a negligible increase in numbers of organisms in the deep flesh of beef stored for two weeks at 41°F., which led Moran (22) to conclude that spoilage by bacteria in the deeper parts of the flesh is unimportant compared with that at the surface. This seems to be the case with packaged meats and, therefore, the following discussion will be limited largely to the nature and extent of the surface contamination.

TYPES OF MICROORGANISMS FOUND

A large number of investigators (1, 2, 5, 6, 9, 11, 14, 21, 28, 34, 35, 44,) have studied the taxonomic distribution of microorganisms

[1] Journal Paper No. J-1926 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1161.

isolated from surfaces of various kinds of meats (*see* Table 1). The following genera are represented: (bacteria) *Pseudomonas, Xanthomonas, Azotobacter* type, *Micrococcus* (*Staphylococcus*), *Gaffkya, Sarcina, Neisseria, Diplococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Corynebacterium,* diphtheroids, *Alcaligenes, Achromobacter, Flavobacterium, Escherichia, Aerobacter, Paracolobactrum, Serratia, Proteus, Salmonella, Hemophilus, Bacterium, Bacillus, Actinomyces, Streptomyces;* (molds) *Rhizopus, Mucor, Zygorrhynchus, Thamnidium, Aspergillus, Penicillium, Sporotrichum, Cladosporium, Alternaria, Monascus;* (yeastlike fungi and yeasts) *Geotrichum* (*Oidium, Oospora*), (*Geotrichoides*), *Candida* (*Monilia, Blastodendrion, Mycotorula*), *Torulopsis,* (*Cryptococcus, Torula*), *Rhodotorula, Debaryomyces,* (*Wardomyces*) and *Saccharomyces.*

INITIAL LOADS OF MICROORGANISMS

There is urgent need for published information regarding the incidence of the several types of aerobic and anaerobic microorganisms found in and on packaged meats. In the past, some question has been raised regarding the value of such observations. Certainly mere counts and taxonomic cataloguing of species encountered on meat does not present sufficient evidence to incriminate or exonerate the product. However, studies which determine not only the numbers of microorganisms present but their contribution to its ultimate spoilage should prove of considerable worth.

According to Haines (11) the greater part of the surface of the whole beef or quarter is covered with a layer of fat and connective tissue upon which little or no bacterial growth occurs. However, the cut-surfaces support the growth of large bacterial populations quickly. This is forcefully illustrated by the very high counts of bacteria on ground meats. Weinzirl and Newton (42, 43) proposed a bacteriological standard of 10^7 organisms per gram after observing that the earlier standard of not over 10^6 suggested by Marxer in 1903 would result in the condemnation of most ground meats. This situation, according to Tanner, results from the fact that since ground meats "may be made from meat scraps and handled carelessly, they are subject to marked bacterial development because grinding thoroughly distributes bacteria, releases juices, and provides a much larger surface for the bacteria."

Initial counts on meats cut or prepared in accordance with sound sanitary practice had relatively low numbers of bacteria when compared with similar items purchased from local retail outlets (*see* Fig. 1).

Generally, packaged frankfurters had very low surface counts (from 20 to 240 per square centimeter). This might be expected since the product: (1) contains bacteriostatic curing agents; (2) is heated sufficiently to destroy many nonspore-forming organisms; (3) has a protective wrap until ready for packaging; and (4) is placed in the final package with minimum handling at the packing plant. These counts contrasted sharply to those obtained from bulk frankfurters

TABLE 1

GENERIC DISTRIBUTION OF MICROORGANISMS ISOLATED FROM SURFACES OF MEATS INVESTIGATOR*

Genus Represented	Product Examined											
	Fresh Beef				Fresh Pork Sausage	Cured Meats	Sausage	Frankfurters	Chicken		Cod	Haddock
	2*	6*	11*	21*	35*	44*	14*	28*	9*	1*	5*	34*
Bacteria												
Pseudomonas		+	+		+				+	+	+	+
Micrococcus		+	+	+	+		+	+	+	+	+	+
Gaffkya				+					+			
Sarcina				+	+		+	+	+	+		
Neisseria					+				+	+		
Lactobacillus							+	+				
Microbacterium					+			+	+		+	
Alcaligenes					+				+	+	+	
Achromobacter		+	+	+	+		+		+	+	+	+
Flavobacterium		+		+	+		+		+	+	+	+
Escherichia					+				+	+		
Aerobacter					+				+	+		+
Paracolobactrum					+				+	+		
Proteus			+		+		+		+	+	+	
Salmonella									+	+		
Bacterium				+	+							
Bacillus				+	+		+	+	+			
Xanthomonas					+							
Diplococcus				+								
Streptococcus			+						+	+		
Corynebacterium									+			
diphtheroids									+			
Hemophilus									+			
Serratia							+					+
Azotobacter type			+									
Streptomyces									+	+		
Actinomyces			+						+			
Molds												
Zygorrhynchus								+				
Mucor	+					+		+				
Thamnidium	+											
Rhizopus	+					+						
Penicillium	+	+				+		+		+		
Sporotrichum	+	+						+				
Aspergillus						+		+				
Geotrichum†		+							+	+		
Cladosporium		+										
Alternaria		+				+		+		+		
Monascus						+						
Monilia‡		+				+		+	+			
Yeasts												
Torulopsis§	+							+	+	+		+
Rhodotorula		+								+		+
Wardomyces‖	+											

* Investigator, see Literature Cited.

† *Oidium*, *Oospora* and *Geotrichoides* included.

‡ *Candida*, *Mycotorula* and *Blostodendrion* included.

§ Also *Torula* and *Cryptococcus*.

‖ Mrak and Bonar isolated *Debaryomyces* from sausage.

(3,100 to 68,000 per square centimeter) for which the handling problem is not nearly so well controlled.

Recent trials (16) with packaged short steak stored at 40°F. indicate a 4-day lag before the onset of off-odor (Fig. 3) when surface counts approximated 185 instead of 32,000 per square centimeter.

Some meats, especially sausages, are heavily spiced. Raw spices contain large bacterial populations and since many of these are spore formers, the heat treatment given the products does not completely sterilize them. Within the past decade precautionary steps, such as the use of ethylene oxide to sterilize spices, have been taken by some

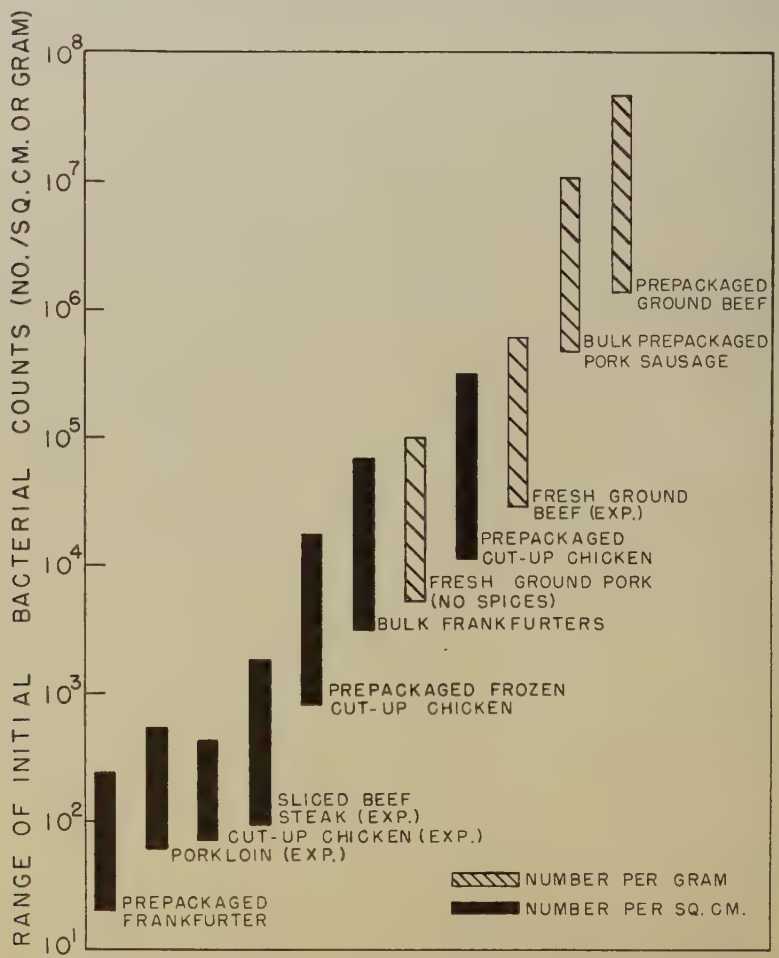

FIG. 1.—Range of initial loads of various fresh, cured, and frozen meats.

processors to eliminate this source of contamination. However, counts made in 1950 (28) of meat from the interior of frankfurters averaged 5,800 bacteria per gram, all of which seemed to be spore formers.

Counts representing one hundred-fold increases have been obtained from packaged ground beef or pork when compared with those found on meats that were ground to order from cheap cuts of meat. It is not

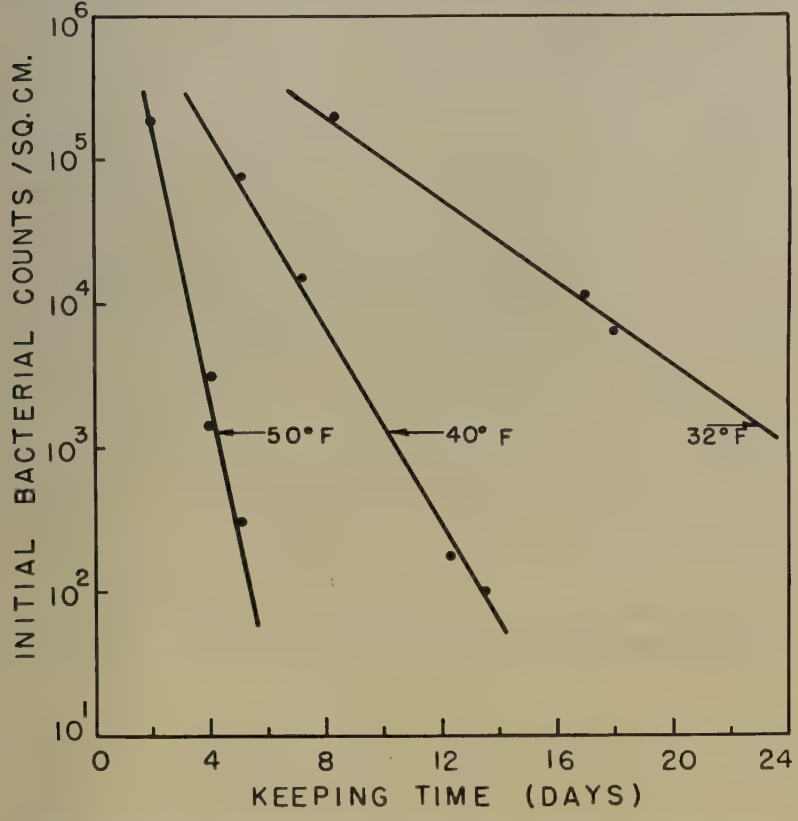

FIG. 2.—Relation of initial bacterial counts to storage life of cut-up chicken at three temperatures. [Ogilvy and Ayres (29); Ayres (unpublished).]

surprising that products with initial loads of from 10^6 to 10^7 organisms per gram undergo spoilage rapidly.

The level of initial contamination has marked influence on the ultimate storage life of meat. Early experiments (28) with cut-up chicken indicated that the storage life was extended by two days at 50°F., by six days at 40°F. and by fourteen days at 32°F. when the starting loads were at a level of 10^3 instead of 10^5 organisms per square centimeter (Fig. 2).

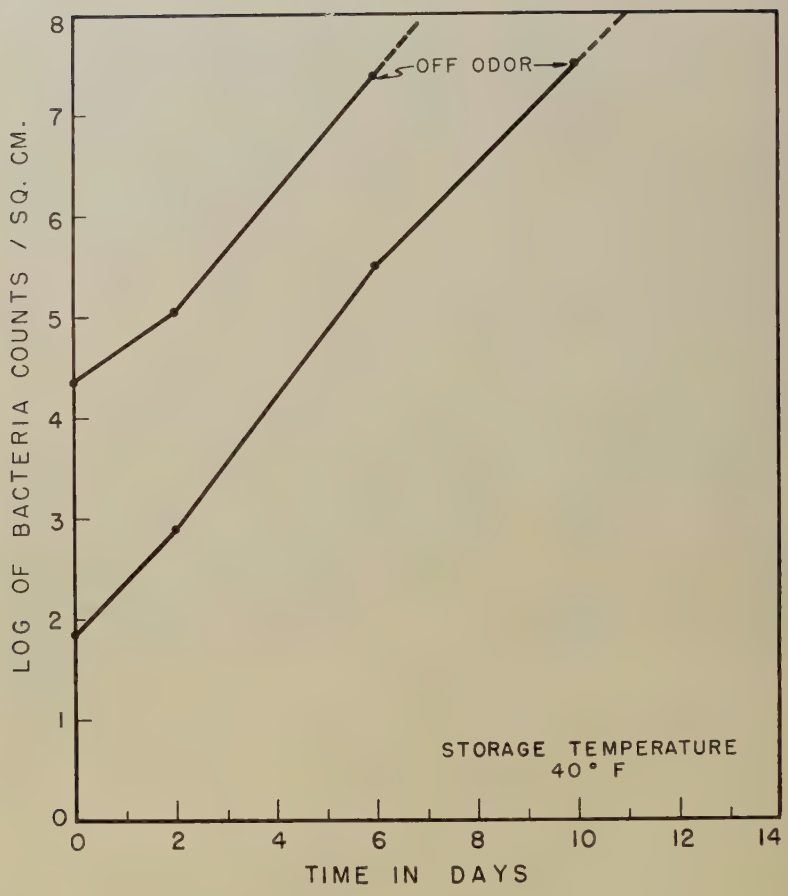

Fig. 3.—Effect of initial bacterial loads on storage life of packaged short steak.

MICROORGANISMS ASSOCIATED WITH SPOILAGE

Although a large number of different kinds of bacteria and other fungi have been found on meats, studies wherein microorganisms have been associated with definite defects revealed the presence of closely related species. Hunter (13) studied the bacterial content of the flesh of salmon and concluded that the forms concerned with decomposition had their natural habitat in the sea water from which the fish were taken. Empey and Vickery (7), Empey and Scott (6), and Mallmann *et al.* (21) decided that most of the organisms comprising the initial flora on beef surfaces had come from the hide and hair of slaughtered animals. A similiar decision was reached by Ayres *et al.* (1) in associating

the contaminants from feathers, feet, and feces of birds with the cut-up flesh.

In the case of fresh animal tissues, a number of workers (1, 7, 11, 22, 29, 32, 34) have indicated that the formation of surface slime on beef, fish, and poultry is primarily due to organisms of the *Achromobacter* and *Pseudomonas* types. As a result of changes in classification made in the more recent editions of *Bergey's Manual,* a number of types previously reported as *Achromobacter,* isolated from slimy fish by Stewart (34) and from slimy beef by Haines (11), no doubt would be classified as members of the genus *Pseudomonas* in the present schema since the organisms in question were reported to have monotrichous flagella. Mallmann (20) has described a slippery condition in dressed squab ducklings in which the incriminated microorganisms were spore-forming capsulated bacilli closely resembling *Bacillus mesentericus.*

That it is not the size alone of the initial contamination which determines the time interval before incipient off-odor or slime points become apparent is shown in Fig. 4. Two samples of ground beef, one prepared from trimmings and stored in the frozen state and the other made to order from chunks of chuck and plate, had almost identical starting loads. Examination of 50 representative colonies isolated from subculture of the frozen ground trimmings (I) indicated 78 per cent to be of the typical spoilage (*Achromobacter-Pseudomonas*) type while but 18 per cent of the isolates from the freshly ground meat (II) was comprised of typical spoilage forms. Many gram positive micrococci, sarcinae, and bacilli and short gram negative rods as well as other miscellaneous forms made up more than 80 per cent of the flora. The two types of comminuted meats were stored in cellophane bags at 40°F. until by organoleptic evaluation each was considered spoiled. The trimmings (I) spoiled in 3 days while the fresh ground beef (II) kept an additional 3 days. At the time of spoilage more than 98 per cent of the flora from both meats was comprised of typical spoilage forms.

In a study of the bacterial flora of fresh pork sausage, Sulzbacher and McLean (35) reported members of six genera to be the most prevalent. They indicated that species of *Microbacterium* made up a rather large proportion of the population of sausage kept at home refrigerator temperatures (41–46.4°F.) and that they may be responsible for the development of the acid taste in stored sausage. The term "lactobacillus" was used by Ogilvy (28) to include members of the genus *Microbacterium, Lactobacillus,* and possibly *Leuconostoc* as well. He doubted whether morphological and cultural characteristics were sufficiently reliable criteria for separating a *Lactobacillus* from a *Leuconostoc* and, further, proposed that the ability of some of the cultures to grow well aerobically might indicate closer affinity to the genus *Microbacterium* than to *Lactobacillus.* Kraft (16) found some of the "lactobacilli" cultures isolated from frankfurters to be catalase positive.

Certain of the unpleasant tastes and odors in fat of stored beef were considered by Lea (17) and by Haines (11) to be caused by microorganisms growing either in fatty tissue or in the adjacent muscle. Lea found that the fat of beef carcasses stored in still air at 32°F. was good after 25 days but somewhat tainted at 42 days; a tainted odor was present at 15 days. Later he (18) stated that tainted fat could contain several million bacteria per gram. Haines (11) and Vickery (39, 40) showed that some of their strains of *Achromobacter* and *Pseudomonas* and all of the yeasts isolated were lipolytic. Coccoid forms — similar to *Alcaligenes viscosus* except that they were almost spherical, nonmotile, and did not produce ropiness — commonly were encountered (1, 29) on chicken that had an off-odor or was slimy. While these organisms were comparatively inert on artificial biochemical media, they showed pronounced ability to hydrolyze fat and so may play some role in spoilage of meat. Of the *Pseudomonas* cultures isolated by Sulzbacher and McLean (35) from fresh pork sausage, 70 per cent were lipase-forming organisms.

Niven (25) obtained evidence that surface greening of stored cured meat was associated with the proliferation of catalase negative *Lactobacillus* and *Leuconostoc*. Ogilvy (28) isolated "lactobacilli" from frankfurters placed in atmospheres containing carbon dioxide; these organisms grew in the presence of high concentrations of the gas in almost pure culture.

Few of the above workers have experienced much success in making positive identification of the several species — and, in some cases, genera — encountered in the various spoilages cited with descriptions listed in *Bergey's Manual*. It is not known whether these various types, which have been associated with animal tissues, exhibit unique characteristics and should be classified as new species or if they are prototypes that give changed reactions upon prolonged artificial cultivation.

Yeasts have been found to be the cause of slime on sausage. Mrak and Bonar (24) isolated the yeast, *Debaryomyces* from slimy sausage.

BACTERIAL LOADS ON SPOILED MEATS

While the distribution of species responsible for spoilage of refrigerated meats seems to be quite restricted, those organisms that do find the environment satisfactory multiply abundantly. Various workers refer to certain minimum concentrations of bacteria at the time that incipient spoilage becomes apparent. Schmid (31) associated the limit of saleability of beef with a bacterial count of 5×10^7 to 10^8 per square centimeter of surface. When bacterial growth is in the advanced stage, meat is slimy to the touch. Haines (11) and Empey and Vickery (7) reported that the slime point was attained when the surface loads were $10^{7.5}$ and 5×10^7 respectively. Lochhead and Landerkin (19) observed odor from New York dressed poultry when counts were in the range 2.5×10^6 to 10^8 per square centimeter. The minimum number of organ-

isms required for sliminess of beef was reported by Moran (22) to be 3×10^6. According to Scott (32) bacterial growth on beef muscle surfaces "only became manifest as slime at relative humidities of 99 per cent and above," and at that time the population (P) was 10^8 or greater. Scott reported:

> At 98 per cent. r.h., growth became visible when $P=5 \times 10^8$ (approximately) but the nodules subsequently did not coalesce to form a slime. At relative humidities of 96.5 and 97 per cent., P attained values of 10^9 without growth becoming visible, except as very small nodules capable of detection only by a lens.
>
> For yeast growth, slime was present at 99 per cent. r.h. when P was between 2×10^6 and 10^7 depending on the size of the individual yeast cells. At 97 and 98 per cent. r.h., growth was manifest as small, transparent, discrete nodules, while at 96 per cent. r.h. these became opaque white. It was also observed that the characteristic odour of spoilage by various bacteria and yeasts was more pronounced on the moist muscle.

Off-odor has been used repeatedly as a method for measuring storage life of meats. Ogilvy (29) obtained good agreement in comparing either off-odor development or slime formation with a definite bacterial load or with an increase in carbon dioxide production. Since the three methods depend on manifestations of bacterial growth, this is as expected.

In the case of cured meats much less work relating to microbial loads at the time of spoilage has been reported. Owing to the strong natural odors of these products, off-odors are not readily detected.

Norton and Roderick (27) believed that micrococci were the microorganisms responsible for slime on sausage. Jensen (14) states that in his own experience, practically every saprophytic mesophile and psychrophile can grow on the moisture film of the casing to form a slime. Niven, *et al.* (26) have presented evidence which indicates that certain types of lactobacilli cause green dicoloration. However, the relationship between lactobacilli and keeping time of stored frankfurters is not yet clearly understood. In Ogilvy's (28) study it was possible to have enormous numbers of lactobacilli without any evidence of greening.

EFFECT OF TEMPERATURE

It has long been known that reduction in temperature results in longer storage life of meats. At temperatures of 32°F. — and even at 40°F. — many mesophilic organisms do not multiply. Empey and Scott (6) found that less than 1 per cent of the microorganisms growing on the surfaces of beef at 68°F. were viable at 30°F. and, although bacteria represented 97 per cent of the contamination acquired by beef surfaces at the higher temperature, yeasts and molds made up a greater share of the population at 30°F. The four principal genera of low temperature bacteria comprising the initial flora were said to be: *Achromobacter,* 90 per cent; *Micrococcus,* 7 per cent; *Flavobacterium,* 3 per cent; and *Pseudomonas,* less than 1 per cent. In an earlier study Empey and Vickery (7) observed that 95 per cent of the initial flora of beef capable of growth at 30°F. consisted of members of the genus *Achromobacter;* the remainder were species of *Pseudomonas* and *Micrococcus.* During

storage the relative numbers of *Achromobacter* and *Pseudomonas* increased while those of *Micrococcus* decreased.

During the first day or two, bacterial counts for meats stored at 40°F. show an initial decline before microorganisms begin to proliferate. (*See* Figs. 4, 5.) Presumably, this temperature may be unsuitable for survival or growth of organisms other than the spoilage types, and insufficient time has elapsed for the psychrophilic organisms to replace these losses.

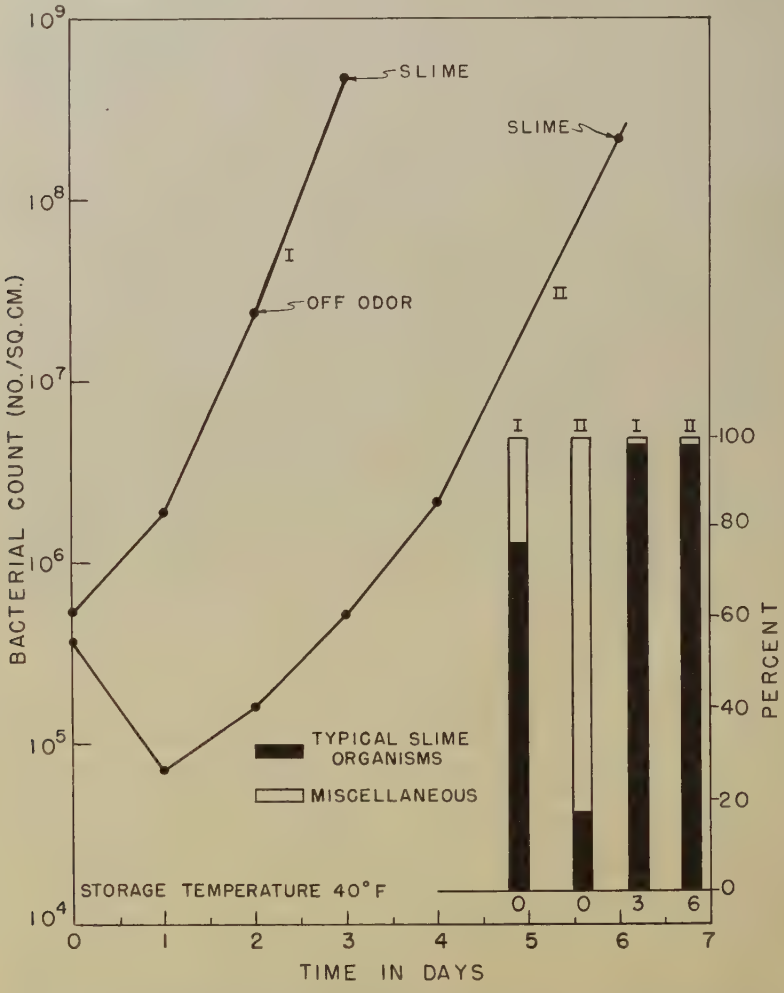

Fig. 4.—Effect of type of initial flora of frozen ground beef trimmings (I) and experimental fresh ground beef (II) on their storage lives. B. Percentage distribution of organisms by type at different storage times.

CRITICAL WATER CONTENT

Walter (41) stated that when relative humidities are less than 96 per cent, bacteria cannot grow at any temperature. This observation was expanded by Scott (32) for specific bacteria and yeastlike fungi

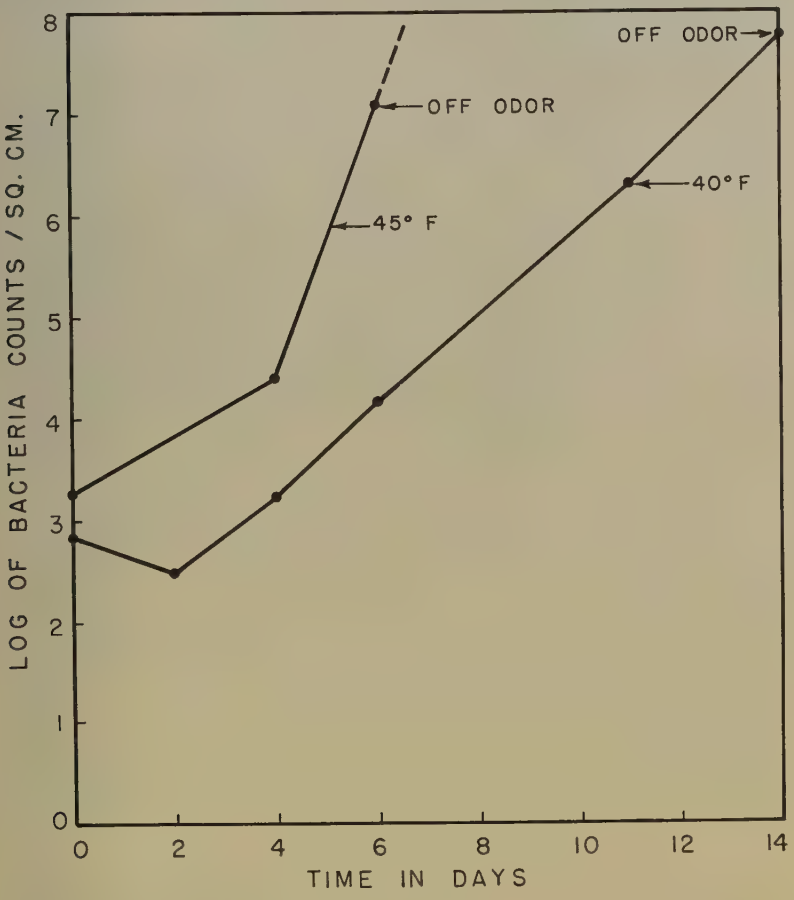

FIG. 5.—Effect of temperature on storage life of packaged round steak.

commonly associated with stored meats. The two *Pseudomonas* strains that he tested were quite susceptible to drying. Their lag periods were extended by five days when the meat was stored at a relative humidity of 99 per cent and one strain, at least, was totally inhibited at 98 per cent. Both types of *Achromobacter* tried were less affected by the dryness of the substrate; their critical range was found to be 96–96.5 per cent r.h. The yeastlike molds *Candida* sp., *Geotrichoides* sp., and *Mycotorula* sp.

grew at relative humidity percentages much lower — 89 to 92 per cent — than those which effectively limited bacterial growth.

Scott (32) found that fresh beef retained its full water content when stored at 99.3 per cent r.h. while Ogilvy (28) estimated the value for cured meat (frankfurter) to be near 98 or 99 per cent. In either case slime-producing bacteria can continue to proliferate if the meat is packaged so that it is sufficiently moisture-vapor resistant to prevent dehydration.

Molds are found to be of primary importance as spoilage agents of cured meats kept under commercial storage conditions. The lower moisture equilibrium and the lower moisture content demands by these organisms are factors which contribute to their predominance over bacteria on unpackaged cured meats or meats packaged with films of high moisture vapor transmission rates. While drying to a point sufficient to prolong the development of a slime point by *Pseudomonas* might be achievable, extreme desiccation would take place before growth of molds would be restricted.

In their present stage of development, cellophane wraps sacrifice some moisture loss in order to maintain good oxygenating conditions. Kraft (16) found an average loss of about 6 per cent in weight from beef packaged with MSAT–80 by the end of the twelfth day in meats stored in a home-type refrigerator.

PACKAGING FILMS

Of the many packaging films available for protecting foods, only a few were compared. Pliofilm FF 120, aluminum-pliofilm laminate (.0015″ Al–120 N 1), Parafilm M, Flexvac, aluminum foil, Saran A 517, and Cry-O-Rap as well as MSAT–80 cellophane were used for packaging fresh beef. Replicate quantities of meats were packaged and, after storage for 0, 2, 5, 8, and 11 days at 40°F. were analyzed. Weight changes as well as bacterial and organoleptic analyses were made. Results of bacterial counts are shown in Fig. 6. Curves for Flexvac, Cry-O-Rap, Saran A 517, and aluminum foil are not shown. Bacterial loads for the first three coincided with those for Parafilm M and Pliofilm FF 120. Counts on the aluminum foil were slightly higher than these but well below those for MSAT–80 cellophane.

The meats packaged with MSAT cellophane developed off-odor or slime more quickly than did any of the other packaging materials. On the other hand the meat in the cellophane packages had best color during the first days of the storage period. Since this film was more permeable to oxygen than any of the others, the colored pigment myoglobin (37) was in the desired oxygenated or scarlet red state.

The aluminum-pliofilm laminate showed least bacterial growth throughout the storage period. The moisture vapor transmissibility of this material is quite low as is also its permeability to gases. Since the foil and film function according to different principles, they have an adjuvant effect. The transmission of moisture vapor through pliofilm

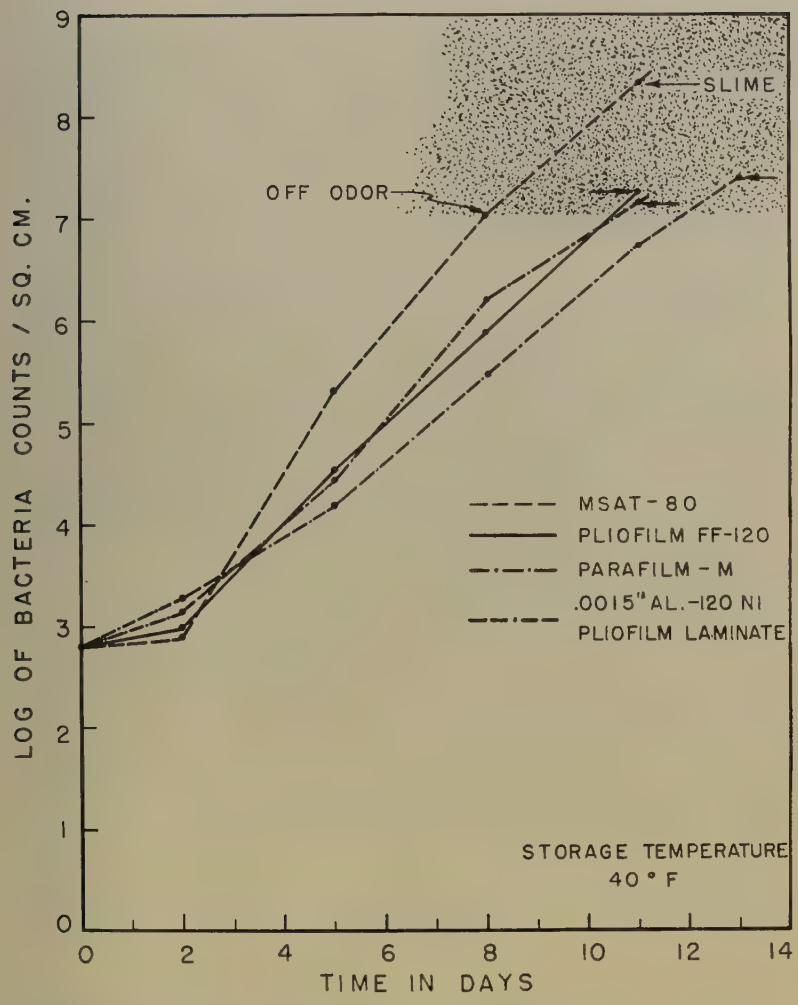

FIG. 6.—Storage life of fresh sliced beef packaged with various films or foils. [Ayres and Kraft (unpublished).]

depends on solution and passage by diffusion but the foil serves as an effective barrier except for minute pinholes. Therefore it is only at the point of pinholing of the foil that transmission of moisture vapor and dissolved gases can take place. It is not known whether accumulation of carbon dioxide or other gases or lack of oxygen is the factor responsible for the decreased multiplication rate. Since these various packaging materials were stored in the dark, no effects due to light

could be recorded for comparing the transparent films with the more opaque materials.

Short exposure of the meat to air prior to packaging did not insure continuance of the scarlet oxymyoglobin. Shortly after placement in the less permeable packaging materials, oxymyoglobin was reduced. However, in the early stages at least, this purple red pigment was readily oxygenated to the desired oxymyoglobin by short re-exposure to air. Prolonged storage resulted in the formation of metmyoglobin in all packaging films. Also, with older samples the moisture-vapor transmission was sufficient with MSAT cellophane so that some discoloration due to local dehydration resulted on the surface of the meat.

While with fresh meats, permeability to oxygen is requisite for the development of proper color within the package, Urbain and Jensen (37) have demonstrated that the pigment of cured meat is discolored by the oxygen of the air. Also, Urbain and Ramsbottom (38) have observed that exclusion of light definitely retards fading.

VACUUM GAS PACKAGING

Vacuum packaging has been suggested (38) as a means for avoiding the harmful effects due to oxygen. Garnatz (8) indicates that this procedure, while materially aiding the color problem, may increase opportunities for bacterial action.

While packaging with an inert gas, such as nitrogen, would accomplish about the same purpose as vacuum packaging insofar as defects due to presence of oxygen are concerned, no positive action against bacterial contamination is exerted. The ideal gas would retard growth of microorganisms without adversely affecting or in any way adulterating the product. While no such gas has been found at the present time, carbon dioxide atmospheres have been shown repeatedly (7, 21, 28, 29) to reduce the rate of proliferation of microorganisms. Their use with fresh meats should not exceed a concentration of 15 per cent if fresh meat color is to be preserved. Since color of cured meats is not adversely affected by the gas, it is with this latter group of products that carbon dioxide may have its greatest applicability. Ogilvy (28) observed that the holding of meats in atmospheres containing carbon dioxide followed by storage in air resulted in longer keeping times for chicken and frankfurters than did air storage alone. He indicated that sufficient carbon dioxide was dissolved in frankfurters by the use of an initial two day exposure in 96 per cent carbon dioxide to give a final 44 per cent concentration of the gas in the atmosphere of a gas tight container wherein the volume of meat and free space were approximately equal. However, gas exposed cured meat spoiled as quickly as did untreated controls when packaged with LSAT cellophane and stored at 40° and at 45°F. This Ogilvy attributed to the failure of the packaging material to prevent loss from the package of the carbon dioxide that diffused from the frankfurters.

Kraft (16) exposed frankfurters to commercial carbon dioxide gas for one day in open Pliofilm FF 120 bags and, after charging the packages with the gas, stored them — as well as controls — at 45°F. The keeping time for the treated samples was 1.5 times that for the untreated frankfurters. Meat that was stored and packaged in air showed incipient spoilage within 16 days while similar products exposed and packaged under carbon dioxide atmospheres before storing in air showed no microbiological spoilage until the 26th day. At these respective times, mold growth was observed. Since it is known (28) that molds are inhibited by low levels of carbon dioxide, it is likely that spoilage of the gas-treated meat took place after most of the carbon dioxide had been lost from the package. Further studies are in progress with other packaging materials.

SUMMARY

Some of the factors which contribute to the microbiological spoilage of packaged meats have been reviewed. A large number of different genera of microorganisms were observed to contribute to the surface contamination of meats. Bacterial counts were found to vary with the product, methods of handling, and degree of sanitation. Finely cut meats were observed to be more heavily contaminated than were larger cuts.

The type of flora encountered — as well as the initial load of microorganisms — had definite influence on the ultimate storage life of the meat. The role of various organisms in causing spoilage was shown to depend upon the temperature of storage.

Preliminary studies conducted using several packaging films and foils indicated that characteristics desired for packaging one type of product were not completely satisfactory for another. While oxygen was required for proper development of fresh meat color, microorganisms grew rapidly on meats packaged in films having good oxygenating properties. Exposing cured meats to commercial carbon dioxide gas prolonged their storage life when they were packaged with materials having low gas permeability.

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MARGINAL RATES OF SUBSTITUTION AND UNCERTAINTY IN THE UTILIZATION OF FEED RESOURCES WITH PARTICULAR EMPHASIS ON FORAGE CROPS*

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Received April 13, 1951

Resource efficiency can be analyzed in terms of aggregative categories of resources such as capital, labor, and land. However, the marginal productivity (in terms of value or other indicators of efficiency or choice) of these various resources can be equated in a manner which maximizes the economic product only to the extent that optimal conditions also exist in use of specific resources within these aggregate categories. In this study, we examine certain relationships for specific capital resources in the form of feed crops. Two important aspects of the feed utilization problem are (1) the marginal rates of substitution of specific feeds and (2) the risk or uncertainty which attend livestock production under different feeding methods. The purpose of this study is to (a) relate basic theory (hypotheses) surrounding these problems with its empirical counterpart and (b) make application of the derived statistics. Thus the investigation is both of *methodological* interest and *practical* application. We present not only the basic hypotheses (models) but also provide detailed steps in empirical estimates and data for livestock feed combination.

This study is exploratory and indeed does not attempt to answer all of the problems in feed utilization. The estimates of this study relate mainly to methodological problems. A very great amount of additional research in animal nutrition is necessary as a basis for refined studies of the nature outlined here. Certainly the physiological and related aspects are great in feed substitution. This study deals only with economic implications of the physical relationships and is a report of preliminary work dealing with feed utilization. This particular summary is made largely to interest and inspire other physical and economic scientists in the problems and methodological procedures employed.

PROBLEM SETTING

A wave of physical experiments is now underway over the nation in an attempt to find means of utilizing additional quantities of forage

* Journal Paper No. J-1867 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1085.

crops. This interest stems from several sources: One is the general public interest in conservation (with more grasses and legumes as one means to this end) and the specific interest of grassland-farming devotees in attaining a goal of all or a major portion of the land area planted to forage crops. Another is the return to production control whereby the farmer is allowed to shift resources from grain to grasses and legumes. Interest is high in the Southeast in the hope that improved varieties and increased production and use of forages will provide the foundation for a prosperous livestock economy for the area. The possibility of war-geared production also gives rise to interest in the degree of substitution and optimum allocation of specific resources. Regardless of the source of momentum, the increased number of investigations of this nature is to be commended. As any production economist is aware, it is the quantitative relationships (physical and value) for the several categories of resources and products which determine their optimum (individual and social) use and combination.

FUNDAMENTAL STRUCTURAL RELATIONSHIPS AND MODELS

This section deals with the models and implications of various types of rotation (primary product) and ration (factor inputs for secondary product) relationships. Readers who are not interested in the basic relationships involved may wish to turn immediately to the statistics derived in the next section. We have previously outlined in detail that when two enterprises such as forage and grain serve on the one hand as primary products and on the other hand as resource inputs for a secondary product such as livestock, output of the latter can be maximized when (a) the marginal rate of substitution of crops as product outputs in the rotation is equal to (b) the marginal rate of substitution of the same crops as feed inputs for livestock production.[1] The determination of these "equilibrium" or "maximum" positions requires that quantitative physical data be available for estimating both the iso-resource curve for crops in rotation and the iso-product curve for crops as feed inputs. These product and resource relationships as well as the maximum conditions outlined above are illustrated in the simple model (hypothesis) of Fig. 1. Curve G_pF_p represents the relationship of the two crops as primary products from land and other resources (the rotation relationships). It is an iso-resource curve suggesting various possible combinations of grain and forage forthcoming from given resources.[2] Curve G_fF_f is an iso-product curve denoting the various combinations of grain and forage that can be employed in producing

[1] For further details on these relationships see:

HEADY, EARL O.
1948. Economics of rotations. Jour. Farm Econ. 30:648–63.

[2] Studies are underway at Iowa State College to estimate the iso-product (livestock ration) and iso-resource (crop rotation) relationship for numerous categories of soils and livestock. Both the (1) fundamental and applied and (2) the individual farm and national policy aspects of these relationships are being examined.

a given (equal) output of livestock product (the ration relationship).[3] Maximization of livestock output from a given land area is hence denoted by tangency G_pF_p and G_fF_f; the marginal rate of substitution of the two crops as product inputs is equal to the marginal rate of substitution of the same two crops as feed inputs.[4]

Because they are prone to believe that greater uncertainty attends the feeding of purchased feeds, many livestock farmers feed the crops in the proportions in which they are produced on the farm (e.g., they

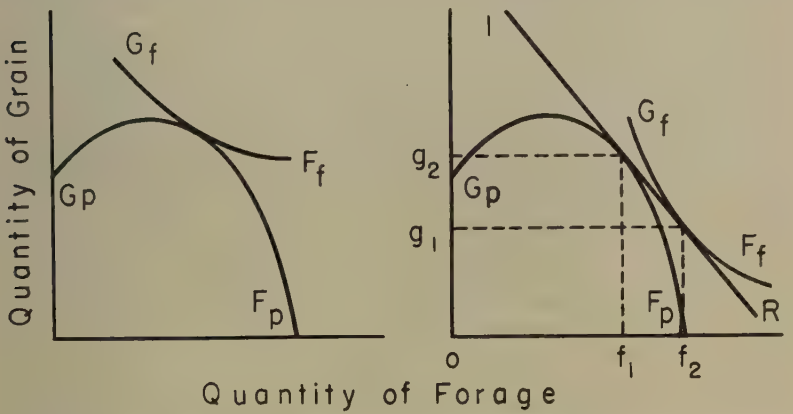

FIG. 1 (left) and FIG. 2 (right)—Hypothetical models illustrating diminishing rates of grain/forage substitution.

neither purchase nor sell feeds). It is also true that farmers in the aggregate must utilize feed crops in the proportions produced (aside from the relatively small quantities used for human food). However, many operators do look upon the feed crop and livestock enterprises as distinct segments of their unit (from the standpoint of maximizing profits). Accordingly, they may attempt to (1) produce crop combinations which will maximize returns to the resources specifically employed and (2) combine feed and other resources in a manner to minimize the cost of producing a specific output of livestock output. Figure 2 provides a simple model illustrating the maximal conditions

[3] There are, of course, an entire family of iso-product contours in production for which G_fF_f would represent one. The complete family is expressed as a production surface.

[4] The portion of G_pF_p with a positive slope is indicative of a complementary relationship between grain and forage while the portion with a negative slope denotes competition between the two groups. The portion with a positive slope is an irrational area of production. For details on these points see:

HEADY, EARL O.
1948. Economics of rotations. Jour. Farm Econ. 30:645–63.

For soils where the relationship is alone one of competition between grain and forage, the iso-resource curve will have a negative slope only. However, the conditions of equilibrium are still exactly as those outlined here.

under this situation. Curve G_pF_p is again an iso-resource relationship of the nature explained earlier while G_fF_f is the iso-product curve. One additional relationship has been added; IR is an iso-revenue line denoting the various combinations of grain and forage which will bring in a given revenue. Its slope is dependent on the ratio of grain and forage prices. Maximum return from grain and forage as primary products sold in the market is denoted by tangency of G_pF_p and IR since at this point the marginal physical rate of substitution of grain and forage is (inversely) equal to the ratio of grain and forage prices. Conversely, IR can be looked upon as an iso-cost curve from the standpoint of livestock production. As an iso-cost curve it shows the various combinations of the two crops which can be purchased with a given outlay of funds and again its slope depends on the price ratios of grain and forage. In this case the minimum cost of producing the given output of livestock (represented by G_fF_f) is denoted by tangency of IR and G_fF_f since again the marginal physical rate of substitution of grain and forage as feed inputs is equal to the ratio of their costs (and the minimum cost or outlay curve is so represented).

For farmers who produce only crops, relationships G_pF_p and IR alone are relevant while for farmers who might produce only livestock (a rare case), G_fF_f and IR are the relevant relationships. The farmer who produces both crops and livestock but as distinct enterprises might also act in the following manner were he to maximize return from both: (a) grain and forage as product outputs should be produced in the proportions of og_2 and of_1 respectively (in order to maximize returns from resources employed in crop production); (b) grain and forage as factor inputs for livestock production should be fed in the proportions og_1 and of_2 (in order to minimize the cost of producing a given output of livestock) and (c) the quantity of grain sold should be g_1g_2 while f_1f_2 forage should be purchased (if returns from the aggregate business are to be maximized).[5]

EMPIRICAL ESTIMATES

In abbreviated terms we have provided the basic logic which indicates the need for and importance of obtaining the product (crop rotation) and factor (livestock ration) relationships. On the pages which follow we explain empirical estimates which are derived for certain livestock feed relationships outlined above. These are confined to dairy cows and hogs and are based on the original data for the Jensen

[5] This assumes that the funds derived from crop production provide those for financing the feeding of livestock. If added funds are available, feeds should still be fed in the proportions indicated if returns are to be maximized. We have attempted to provide the basic logic by means of some "pure" cases. Obviously there are numerous variations of and exceptions to these. However, the basic relationships which we have presented here are necessary data if information is to be made available to farmers in a manner which allows them to determine the most efficient combination of resources.

input-output study[6] and the Beltsville experiment[7] respectively. It should be emphasized that the empirical estimates which follow appear to be about the best which can be derived from available experimental data. After making a rather wide survey of the physical input-output data available, we decided that the two studies cited provide information which best lends itself to the grain-forage substitution relationship. While the Beltsville experiment was designed to provide data on substitution rates, the Jensen study was directly related to levels of grain feeding for dairy cows and only indirectly to substitution rates. However, since higher levels of grain feeding were possible only as levels of hay feeding were reduced, the resulting data included an element of feed substitution as well as a pure grain/milk input-output relationship.[8]

The possible limitations of the Jensen data in predicting substitution relationships (as well as direct input-output relationships) can be illustrated by reference to the hypothetical example in Fig. 3. Here the process of milk production is represented as a production surface. The vertical or Y axis is taken to represent milk output per cow while the "left hand" or X_1 axis represents the amount of hay fed and the "right hand" or X_2 axis represents the amount of grain fed. Thus milk output might be held at some fixed level such as that indicated by the contour ab. This same level of milk production (say 8,000 pounds per cow) might be attained by feeding more hay and less grain (movement from the right to the left on contour ab) or more grain and less hay (movement from the left to the right on ab). An experiment in dairy production designed to isolate substitution rates per se would attempt to hold milk output per cow constant (at ab for example) and simply "move around" ab. An experiment designed to get at the grain-input and milk-output relationship alone would hold hay per cow at some fixed level (as that indicated at b) and "move up" bd. An experiment could also be designed (but would be extremely difficult and costly) which would attempt to estimate that portion of the production surface indicated as $abdc$ in Fig. 3. This would be accomplished by holding milk per cow constant at several levels (such as ab or cd) and varying the quantity of grain and hay for each of these levels.

The Jensen study (including all cows) was not primarily designed to isolate any of the above relationships. Rather it more nearly traced out a line such as ad in Fig. 3. This line partly confounds the grain-

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[6] Jensen, Einar, *et al.*
1942. Input-output relations in milk production. U.S.D.A. Tech. Bul. 815.

[7] Ellis, N. R., J. H. Zeller, and J. K. King.
1943. The value of good legume hays in the ration of fall pigs. U.S.D.A. A.H.D. No. 60.

[8] Economists will recognize that we are attempting to derive the factor-factor relationship in production while the Jensen experiment was designed more nearly to isolate the factor-product relationship. The study did, however, interrelate the factor-factor and the factor-product relationships in the manner to be outlined.

forage substitution relationship. Starting from the standard Haecker ration (which might be indicated as point *a* in Fig. 3) cows were fed greater amounts of grain and were allowed to make their own selection of forage intake. Two things happened as higher levels of grain were fed: (1) Less hay was consumed per cow. (2) More milk was produced from each cow.

It is perhaps true that in terms of costs and with the inclusion of all factors the line *ad* is the important one in forage utilization through dairy cattle. Attainment of a line such as *ab* or *cd* would necessitate that both grain and forage be weighed or measured carefully. Thus the cost of the added labor for this operation might amount to more than

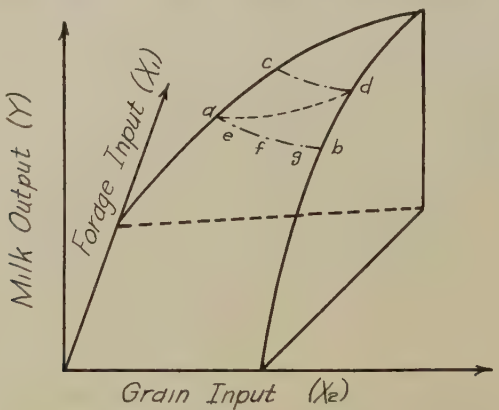

Fig. 3.—Production surface illustrating substitution and transformation relationships.

the additional feed costs which result if the cow is fed a limited amount of grain and allowed free choice of forage (and a movement up or down line *ad* takes place). The labor problem is not so important for other types of livestock. In any case the rate of grain-forage replacement is important in today's farming problems.

The sample of cows which we selected from the several hundred in the Jensen study appeared to give a fair scatter over an entire "slice" (such as *abdc*) of the production surface. However, we cannot be certain that confounding of the two relationships (factor-factor and factor-product) has been entirely eliminated.[9] The production function (the production surface or a portion of it such as *abdc* in Fig. 3) and the product contour or substitution line (the forage grain line such as *ab*) have been derived for milk production in the manner to be outlined.

[9] Actually the "summit" of the production surface in milk production may more nearly take the form of *ad* in Fig. 3. Because of the limited capacity of a cow's stomach, maximum milk output per cow must necessarily vary with the proportions of different feeds in the ration.

The problem of deriving substitution rates in pork production is somewhat different. Pigs started at the same weight (such as on contour line *ab* in Fig. 3 when Y represents pork output or weight per pig) and finished at the same weight (as on contour line *cd*) allow an estimate of an entire portion of the production surface (such as *abdc*) when several different grain-forage rations (such as *a*, *e*, *f*, *g* and *b*) are fed. However, unless feed inputs are available for different weights (rather than for only beginning and final weights), estimates can be made only for the average rate of substitution over an entire slice of the production surface (rather than for one particular level on the surface such as represented by *ab*). Since the data available are in the form of beginning and final weights only, it has also been necessary to employ a different statistical technique for pork as compared to milk substitution rates.

Detail on derivation of substitution rates is given below. Again it is our hope that these data (1) may be of some practical use (given the current paucity of experimental data from which substitution rates can be derived, (2) can serve to generate additional effort in design of experiments and empirical techniques which will allow estimate of substitution rates. With the great emphasis now being placed on grassland farming, the problem is of important magnitude.

THE PRODUCTION FUNCTION

The production function (relationship between input and product output) for livestock is of the general form $Y = f(X_1, X_2, X_3, X_4, X_5, X_6 \ldots X_n)$ where Y represents the output of product, while the inputs are forage, grain, protein supplements, labor, other capital, time, and other specific resources.[10] Cattle fed on grass (more forage) may require less of both labor and grain than cattle fed in dry lot (less forage). Grain is not only a substitute for forage in producing meat but also is generally a substitute for time (a shorter time span is required for an animal fed a high-grain ration than for one fed a high-forage ration in attaining a given output of product of specified grade). Thus final solution of the forage utilization problem depends on establishing the nature and rate of substitution of various forages for time, labor, and other capital resources in addition to grain.

SUBSTITUTION RATES IN DAIRY PRODUCTION

Our first derivations are for dairy production. The procedure involves three steps in derivation of (1) the production function, (2) the iso-product equation (the combination of grain and forage to produce a given quantity of milk product) and (3) the marginal rate of substitution of hay for grain (the amount of grain replaced by each successive pound of hay with output constant). The estimates are based on

[10] These are broad catagories or aggregates of resources and might (should) be further classified. For example X_1 (forage) might be further subdivided into x_1 (alfalfa), x_2 (no. 2 grade clover), x_3 (bluegrass pasture), x_4 (no. 4 grade clover), x_5 (brome grass silage).

observations for thirty individual cows over a year's time from the Jensen experiment. We have employed only series II data (grain ration varied with free choice of hay) for all heavy-breed cows (Holstein and Brown Swiss), receiving comparable feeds (hay, corn silage, and grain) all with an expected output-capacity of 300 to 400 pounds (when fed the standard Haecker ration). Pasture was converted to a hay-equivalent and silage was broken down into a grain and a forage component.[11] Because of the complexity of the analysis and the paucity of data only feed inputs have been included in estimates of the production function.[12] We thus make estimates of the forage-grain product contour (curve G_fF_f in Fig. 1) from a production function which now takes the form $Y = f(X_1, X_2)$. (Y refers to milk output while X_1 refers to forage input and X_2 refers to grain input per cow.)

In our initial work we employed six related but distinct production functions (regression equations):

(1) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2$

(2) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_1 X_2$

(3) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2$

(4) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2$

(5) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_2^2$

(6) $Y = \alpha + \beta_1 X_1 + \beta_2 X_2$

(7) $Y = \alpha X_1^{\beta 1} X_2^{\beta 2}$

The first production function seemed a logical hypothesis since the squared terms allow a diminishing rate of transformation of each feed into milk while the X_1X_2 terms not only allow diminishing transformation but also allow interaction between feeds when either is fed at a high level (while the other is fed at a low level). The same possibilities existed in the second production function. The third allows diminishing transformations of each feed. The fourth and fifth each allow diminishing returns to one feed, with a condition of linearity for the other. The sixth equation tests the hypothesis of a completely linear production function while the seventh allows constant elasticities but diminishing productivities.

The first (1) production function is acceptable in terms of statistical probability but not in terms of production logic. The positive coefficients

[11] While not strictly correct since it assumes a constant rate of substitution between hay and forage from silage and between straight grain and grain from silage, components of silage were converted in terms of their energy replacement value on the basis of Morrison standards as presented in:

Morrison, F. B.
1948. Feeds and Feeding. 8th ed. 1207 pp. Morrison Publishing Company, Ithaca, N. Y.

[12] While this restricted analysis allows consideration of the economy of two specific feeds, it does not allow efficiency measurements when other resources are not scarce. The simple production function is alone relevant only when all other resources are fixed or free in the sense that costs are not attacked. We have derived production functions including time as one variable in livestock production as related to different rations. While extremely important, space does not permit a presentation of this data.

for the squared and interaction terms cause increasing rather than decreasing returns. All other equations gave decreasing returns (except the linear one). The seventh (7) production function appears most nearly acceptable in terms of production logic and statistical probability.[13] We present the derived production functions below for equations 1 and 7 with Y as milk and X_1, and X_2 as forage and grain, respectively. Feed is measured in hundred weight for 1 and in pounds for 7.

$$(1)\quad Y = 352 - 4.8434X_1 - 5.937X_2 + .019015X_1^2 + .022887X_2^2 + .057101X_1X_2$$

$$(7)\quad Y = .5513X_1^{.5035}X_2^{.4000}$$

Had production function 6 alone been acceptable (the coefficients for X_1 and X_2 were both significant at the 1 per cent level of probability) the implications would have been these: Feed and grain substitute at constant marginal rates and hence feed costs per 100 pounds of milk can always be minimized by feeding grain alone (no hay) or feeding hay alone (no grain) except when the grain/hay price ratio is equal to the (constant) marginal rate of substitution. Costs would then be equal for any possible combination of hay and grain, and economic limits to level of output per cow would not exist. These conditions are not consistent with the logic of production economics and previous findings in animal nutrition. Production function 1 implies none of these conditions as will be pointed out later.

ISO-PRODUCT CONTOURS OR FEED COMBINATIONS

Equations which represent the empirical counterpart of the iso-product contours (G_fF_f) in Figs. 1 and 2 can be derived for each of the production functions. These allow milk output to be held constant while the values of X_2 (grain) are solved for the various values of X_1 (hay) and can be used to derive the amount of grain associated with each level of hay feeding. This indirect approach to the contour (feed combination) equation was employed since it not only conforms to production logic but also eliminates the need for considering either feed as the independent variable (and hence obtaining either one of two curves, depending on the direction, errors are minimized) were the equation derived directly. By algebraic simplification the iso-product contours thus become as follows for production functions 1 and 7 with milk output at 8,500 pounds per cow.

$$(1)\quad (8{,}500)\ X_2 = \frac{1}{.045774}\ (5.4937 - .057101X_1) \pm \sqrt{5.7393 - .1840X_1 + .001520X_1^2}$$

$$(7)\quad (8{,}500)X_2 = \left(\frac{8{,}500}{.5513X_1^{.5053}}\right)^{1/.4}$$

[13] The t values for the β's of production function 1 are (a) 2.109619, (b) 2.340752, (c) 1.976709, (d) 2.278925, and (e) 3.065689 (in order of their sequence in the regression equation). The t values for equation 7 are 2.29 and 2.49.

It is now possible to derive equations for the marginal rates of substitution of hay and grain. These equations (listed below for 8,500 pounds of milk per cow) are the first derivatives of the contour equations and indicate the rate of change in X_2 (grain) for each one unit change in X_1 (hay).

$$(1)\ (8{,}500)\ \frac{dX_2}{dX_1} = \frac{-.1840 + .003039X_1}{.0915\ \sqrt{5.7393 - .183984X_1 + .001520X_1^2}} - 1.2475$$

$$(7)\ \ (8{,}500)\ \frac{dX_2}{dX_1} = \frac{.5035X_2}{.4X_1}$$

EMPIRICAL ESTIMATES AND INTERPRETATION

By setting the quantity of hay at different values (column 1) for production function 7 we have solved for the quantity of grain in producing 8,500 pounds of milk (from the contour equation). These are presented (column 2) in Table 1. The marginal rates of substitution (the equation representative of the first derivative) are given in column 3 while the reciprocal in column 4 indicates the pounds of hay required to replace each pound of grain (with milk output constant at 8,500 pounds per cow for the year) for each combination of feeds. The level

TABLE 1

GRAIN-FORAGE ISO-PRODUCT CONTOUR (FEED COMBINATIONS) AND MARGINAL RATES OF GRAIN-FORAGE SUBSTITUTION FOR MILK OUTPUT OF 8,500 POUNDS

Feed Combination for Producing 8,500 Pounds of Milk (Iso-Product Contour)		Marginal Rate of Substitution of Hay for Grain $\frac{dX_2}{dX_1}$ (Pounds of Grain for Which 1 Pound of Forage Substitutes) *	Pounds of Hay Required To Substitute for 1 Pound of Grain †
Lbs. of Hay X_1	Lbs. of Grain X_2		
(1)	(2)	(3)	(4)
5000	6154	1.55	.65
5500	5454	1.25	.80
6000	4892	1.02	.97
6500	4423	.86	1.17
7000	4029	.72	1.38
7500	3694	.62	1.61
8000	3406	.54	1.87
8500	3156	.47	2.14
9000	2937	.41	2.43
9500	2744	.36	2.75
10000	2572	.32	3.09
10500	2419	.29	3.45
11000	2281	.26	3.83
11500	2157	.24	4.24
12000	2096	.22	4.55

* The marginal rates of substitution refer to exactly the points on the contour indicated in columns 1 and 2. They are not averages between points.

† Column 4 is the reciprocal of column 3.

of 8,500 was selected because it represented an observed output from the greatest number of cows. The extreme ranges of hay used in Table 1 are within the observed ranges for cows producing near the 8,500-pound level.

The production function employed in deriving the estimates of Table 2 implies diminishing returns to feed inputs irrespective of the ration fed (and hence increasing marginal costs as output is extended to higher and higher levels). More important for the problem here, the derived statistics indicate a diminishing marginal rate of substitution of forage for grain for any one level of output. The implications of our contour are these: Feed costs can be minimized (for a given output of milk per cow) by a grain-forage ration wherein the ratio of grain/hay prices (or the cost ratios if the feeds are to be produced on the farm) is equated (inversely) with the marginal rate of substitution of hay for grain. With milk output at the rate of 8,500 pounds per cow, and with grain at 1.87 cents per pound and hay at 1.0 cent per pound (a ratio of 1.87:1.0), feed costs per 100 pounds of milk are at a minimum with a ration including 8,000 pounds of hay and 3,406 pounds of grain (where hay substitutes for grain at the ratio of 1.0: 1.87). A grain/forage price ratio of 1.17:1.0 would specify a ration of 6,500 pounds of hay and 4,423 pounds of grain for minimum costs.

If forage substitutes for grain in the rotation at a constant rate of 3.83 pounds of hay to 1.0 pound of grain, milk output (with each cow producing 8,500 pounds) can be maximized from a given land area with a ration including 11,000 pounds of hay equivalent 2,591 pounds of grain. A rotation substitution rate constant at .97 hay: 1.0 grain specifies that a ration of 6,000 pounds of hay and 4,892 pounds of hay will maximize milk production from a given land area (since .97 pound of hay are required to replace 1 pound of grain with this ration). If grain and forage substitute at a diminishing rate (with or without a complementary range) in the rotation, then the marginal rate of grain-forage substitution must be equated with their marginal rate of substitution in the dairy ration if milk output is to be maximized from a given land area.

Product contours with milk at 8,500 and 9,500 pounds per cow are shown in Fig. 4. These contours represent single vertical points on the total production surface (two of a family of contours). Their slopes differ depending on the level of milk production per cow.[14] They are based on equation 7.

OTHER NOTES ON DERIVATION

Data have been derived only for feed and output levels falling

[14] While any one of an entire family of contours (vertical points on a total production surface or feed combinations for various outputs per cow) can be derived from the data, inspection of the original observations and derived estimates suggest that the regression equation has meaning only for contours representing an output of 8,500 to 9,500 pounds of milk per cow. Cows in the experiment not only consumed feeds in the proportions indicated in the range of Table 1 but the milk output per cow fell mostly within 8,500–10,000 pound range.

within the observations of the experiment. The observations fall more in the direction of high grain rather than high forage (the purpose of the original experiment). Forage production problems can only be solved as wider ranges of observations are available at greater extremes in feed combinations. The data employed provide an estimate over a very narrow horizontal "slice" or "band" of the total production surface. While the data for all cows in the experiment perhaps represent a "diagonal movement" over one horizontal slice of the surface (from the bottom of the high-forage extreme to the top of the high-grain extreme), the particular sample of cows used in the analysis evidently represented a great enough variation over the entire slice to allow significant regression coefficients for the original production function. However, to the extent that vertical "movements up" the production surface are confounded with "movements around" the surface in the estimates derived, final determination of substitution rates will have to await other experiments wherein the attempt is made to isolate contour lines rather than capacity lines. (See discussion of later sections.)

ISO-QUANTS (PRODUCT CONTOURS OR FEED COMBINATIONS) AND SUBSTITUTION RATES IN PORK PRODUCTION

While dairy cows were investigated as a type of livestock for which forage-grain substitution might be high, data for hogs are investigated in this section since they might fall at the other extreme. The derived pork iso-quants are based on six experiments in two years with forage ranging from 0 to 20 per cent of the ration. Hogs went into the experiments at approximately equal weights and were fed to 225 pounds. Three forages (alfalfa, sericea lespedeza, and soybean hay) grading U. S. No. 2 or equal were tried separately as ground hay fed in dry lot each year (along with corn and supplemental feeds of a specific grade and quality).

In analysis of the data we have gone directly to the equations for the iso-quants and have eliminated the production function step employed for dairy cows. In deriving the iso-quants (feed combinations in producing equal quantities of pork) we have tried the following equations (where X_2 refers to the pounds of grain and X_1 refers to the pounds of hay consumed per 100 pounds of pork produced). These were derived for each of the three types of hay separately and for all three pooled into one set of aggregate observations.

$$(7)\quad X_2 = \alpha + \beta_1 X_1$$
$$(8)\quad X_2 = \alpha + \beta_1 X_1 + \beta_2 X_1^2$$
$$(9)\quad X_2 = \alpha + \beta_1 X_1 + \beta_2 X_1^3$$
$$(10)\quad X_2 = \alpha + \beta_1 X_1 + \beta_2 X_1^2 + \beta_3 X_1^3$$

The cubed coefficients (9 and 10) were not acceptable at even the 20 per cent level of probability for any of the hays. The squared term (8) approached significance at the 10 per cent level of probability for the soybean, sericea lespedeza, and the pooled data. The linear term

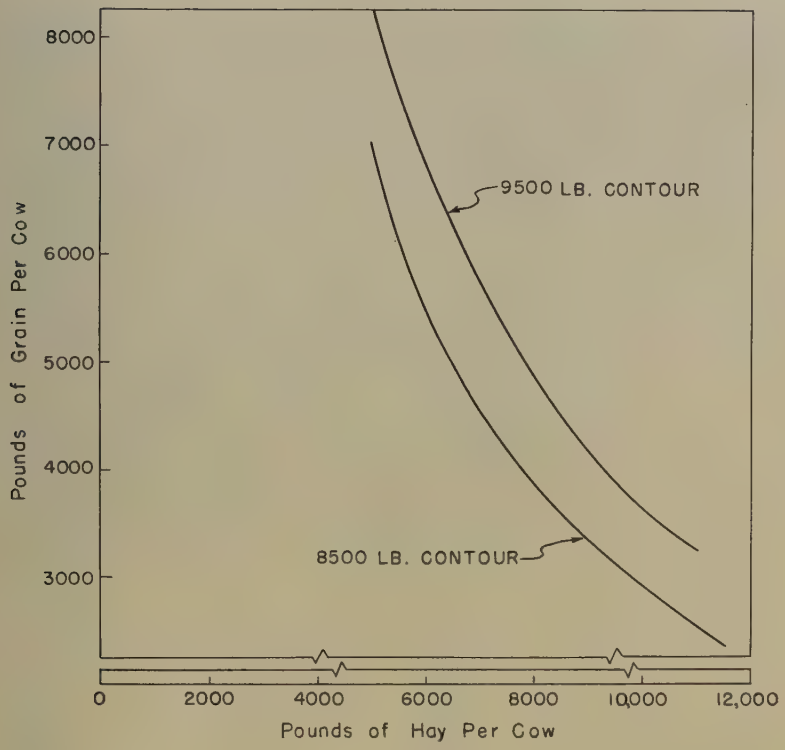

FIG. 4.—Product contours (feed combinations) at 8,500 and 9,500 pounds of milk per cow. (Derived from experimental data with production function 7.)

for this equation (8) was acceptable at the 5 per cent level of probability for the same three feeds (lespedeza, soybeans, and pooled data). The coefficients for the pork iso-quants (feed combinations in producing 100 pounds of pork) are thus given below. (Again these become the empirical counterpart of curve G_fF_f in Fig. 1.)[15]

Sericea lespedeza:	$X_2 = 327.5 - .5113X_1 + .00423X_1^2$
Soybean:	$X_2 = 349.2 - 1.6985X_1 + .01340X_1^2$
Pooled data:	$X_2 = 333.2 - .9860X_1 + .00733X_1^2$

[15] For the linear equations alone (7), the regression coefficient was significant at the 20 per cent level of probability for lespedeza, at the 10 per cent for alfalfa and at the 5 per cent level for soybean and pooled data. The linear regression equations were (a) pooled data: $X_2 = 326.3 - .3559X_1$, (b) Alfalfa: $X_2 = 320.8 - .4406X_1$, (c) lespedeza: $X_2 = 323.2 - .1310X$ and (d) soybean: $X_2 = 336.7 - .5520X$. Acceptance of linear substitution rates would specify the following constant (average and) marginal rates of substitution: (a) Pooled hays, —.3559; (b) alfalfa, —.4406; (c) lespedeza, —.1310; (d) soybean, —.5520.

In contrast to the derived coefficients for dairy, the pork iso-quant equations (combinations of feeds in producing 100 pounds of pork) above refer to an average over approximately the 65–225 pound weight range (the average over a horizontal slice of the production surface). The substitution figures for dairy referred to one level of output (one vertical point on the production surface). Application of the method used for dairy cows was not successful in analysis of the hog experiments (perhaps among other things) because of the small number of observations.[16]

If the above pork iso-quants are accepted, the marginal rates of forage grain substitution can be derived in the conventional manner. These are again given by differentiation in the form of first derivatives (and indicate, at each point of forage feeding, the pounds of grain for which one pound of hay substitutes).

$$\text{Lespedeza: } \frac{dX_2}{dX_1} = -\ .5113 + .00846X_1$$

$$\text{Soybean: } \frac{dX_2}{dX_1} = -1.6985 + .02680X_1$$

$$\text{Pooled: } \frac{dX_2}{dX_1} = -\ .9860 + .01466X_1$$

Data are derived in Table 2 for the pork iso-quant or contour (indicating the various combinations of grain and hay in producing 100 pounds of pork) and the marginal rates of substitution. The derived pork contour or iso-quant is also illustrated graphically in Fig. 5.

On the basis of the estimates provided above, the rate at which forage substitutes for grain is considerably greater in milk than in pork production (to the extent that the techniques of estimation allow comparisons). It is true, of course, that the rate of substitution varies with each kind of forage as well as its quality.[17]

Were the linear contour and substitution equations accepted for

[16] There were only ten observations or degrees of freedom for each single feed and thirty for the pooled data. Undoubtedly the number of observations was too small (each hay taken alone) for a regression equation including four to six variables. This is also somewhat a problem for the three variables (X_2, X_1, X_1^2) employed in the simple analysis above. In one sense there is logic to support our method (under the experiment, various levels of hay were fed to determine grain "requirements" for each). However, the technique is open to question since a different regression curve (and hence marginal rates of substitution) is obtained depending on which feed is termed the "independent variable" and hence on the direction in which errors are minimized.

[17] For example the pounds of soybean hay required to substitute for each pound of grain (the parallel of column 4 in Table 2) are as follows (with the level of hay feeding in parentheses): (0) 1.01, (5) 1.10, (10) 1.19, (15) 1.31, (20) 1.44, (25) 1.61, (30) 1.83, (35) 2.11, (40) 2.50, (45) 3.06, and (50) 3.95.

lespedeza, the implications would be these: (1) Forage and grain substitute for each other at the constant rate of .1310 pound of grain for each pound of hay (7.64 pounds of hay are required to substitute for each pound of grain) regardless of the amount of hay fed. (2) Were feed costs per 100 pounds of pork to be minimized, (a) no hay should be fed unless the price of grain per pound is more than 7.64 times the price of hay per pound and (b) all hay should be fed when the hay: grain price ratio is more than 1.0: 7.64.[18]

The implications are entirely different, however, if the equations for the concave product contour and diminishing marginal rates of substitution (feed combinations and substitution rates of Table 2) are

TABLE 2

PORK ISO-QUANT OR PRODUCT CONTOUR (FEED COMBINATIONS FOR PRODUCING AN EQUAL OR 100-POUND OUTPUT OF PORK). LESPEDEZA HAY AND GRAIN

Feed Combinations for Producing 100 Pounds of Pork (The Pork Iso-Quant)		Marginal Rate of Substitution of Hay for Grain $\frac{dX_2}{dX_1}$ (Pounds of Grain for Which 1 Pound of Hay Substituted) *	Pounds of Hay Required To Substitute for 1 Pound of Grain †
Lbs. of Hay X_1	Lbs. of Grain X_2		
(1)	(2)	(3)	(4)
0	327.5	.5113	1.96
5	325.1	.4690	2.13
10	322.8	.4267	2.34
15	320.8	.3843	2.60
20	319.0	.3420	2.92
25	317.4	.2997	3.34
30	316.0	.2574	3.88
35	314.8	.2151	4.64
40	313.8	.1728	5.79
45	313.1	.1305	7.67
50	312.5	.0881	11.35

* The figures refer to substitution at the exact level of hay feeding indicated in column 1.
† Column 4 is the reciprocal of column 3.

accepted. Here minimum feed costs per 100 pounds of pork vary between specific combinations of grain and forage depending on the ratio of prices. With a forage/grain price ratio of less than 1.0: 1.96, a ration which will minimize feed costs will contain only grain. A forage/grain price ratio of 1.0: 2.60 would specify 10 pounds of forage and 322.8 pounds of grain while a price ratio of 1.0: 5.79 would specify

[18] We refer only to feed costs. It is true, of course, that forage is complementary to time and hence any greater labor requirements or lower sasonal prices associated with high-forage rations must be considered in figuring net returns. These and other cost elements are included in the data of later tables.

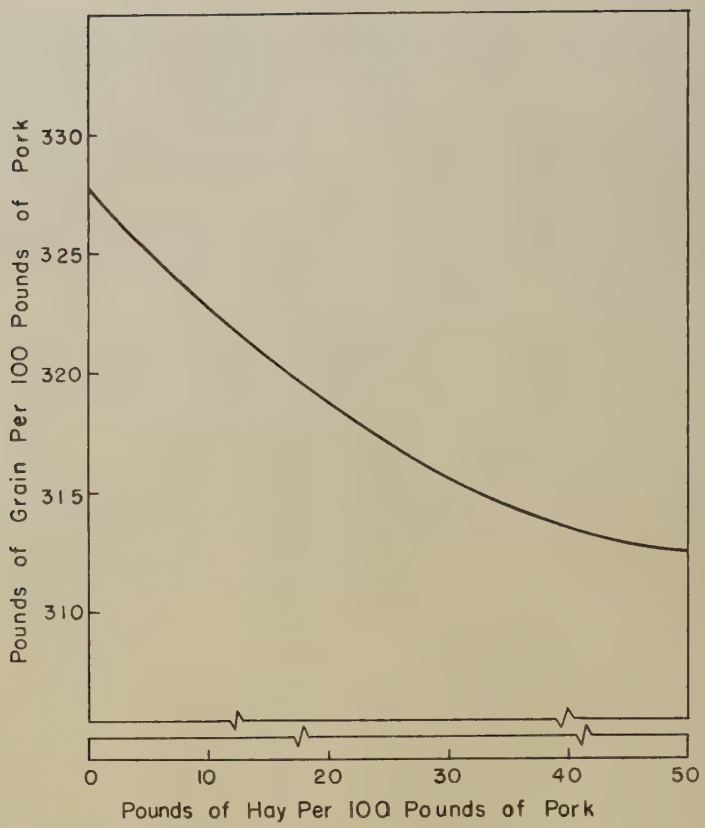

FIG. 5.—Feed combinations for producing 100 pounds of pork (average product contour derived from experimental data with contour equation 2).

40 pounds of hay and 313.8 pounds of grain. If forage and grain serve competitively in the rotation, maximum pork production per acre of land would come (a) with all land planted to hay if forage in the rotation always substitutes for grain at a rate of 11.35:1.0 or greater. (b) With all land planted to hay if the forage-grain substitution ratio is always less than 1.96:1.0 or (c) with a rotation which combines hay and grain in a manner to equate substitution rates in crop production with those of the livestock ration (e.g., as at forage-grain substitution ratios of 3.34:1.0, 2.34:1.0, etc.). The (a) and (b) conditions also apply when crops in rotation are complementary.[19]

[19] The substitution rates are for grain and forage only. We have made estimates of forage substitution relative to other feeds as well as grain. These are not presented for lack of space. However, since the amounts of other feeds (tankage, mineral, salt, etc.) were held constant in the experiments, these coefficients do not change the general conclusions.

FEED COMBINATIONS AND MARKET UNCERTAINTY

The analysis above has been entirely in the vein of minimizing the cost (in terms of value of feeds or input of land) of producing a given output of livestock. An added variable enters the problem, however, when market returns for the product are considered. As already indicated, forage and time generally are complementary factors of production since a longer period is required in producing a pound of livestock products by means of forage rather than grain. (Grain substitutes for both forage and time in meat production.) Thus the price and time variables become interrelated in two respects: First, the marketing of the product may fall into a (known) period of lower (or higher) seasonal prices as the production period is extended. Second, because of the greater time span of the production period, risk or uncertainty may be increased. The latter possibility exists in the sense that the probability of deviations in commodity or resource prices from expectations (upon which plans are based) increases as the production period is extended. Problems of the first nature (seasonal prices) can be handled partly through the timing of the enterprise (farrowing pigs earlier, putting cattle on grain feed sooner, etc.). Extension economists put forth the hypothesis that is the risk (uncertainty) which perhaps should also serve as a criterion in judging the efficiency of alternative feed utilization systems. Accordingly, this section is devoted to an analysis of the degree of risk or uncertainty which attends the substitution of forage for grain feeds. We believe that the problem posed is important: Farmers are perhaps as greatly concerned with the degree of uncertainty surrounding alternative investments as in mean returns over time. While it may be known that alternative *B* will return only one-half as much as *A* over a twenty-year period, a farmer with limited capital and concerned with business survival over the next five years may well select *B* over *A* if the risk or uncertainty surrounding the former is less than for the latter. (He may wish to select an enterprise with a low mean return over time if variations in returns are also low rather than one with high average returns over long periods but with wide fluctuations in short periods.) How does the greater time period required under heavy forage feeding affect risk or uncertainty (variability) of income?

MEASUREMENT OF UNCERTAINTY

The degree of uncertainty surrounding an economic venture is expressed in the skewness, kurtosis and several measures of dispersion (range, variance, standard deviation, coefficient of variation) attached to the particular income probability distribution in question. The relationship of each of these characteristics of the probability distribution to the degree of certainty attached to alternative ventures can be expressed in abbreviated terms by means of Fig. 6. Suppose that the hypothetical probability distributions represent the expected (or the historic) outcome for five distinct enterprises. The modal or most likely income is

identical since the probability is greatest for Z return in all cases (except E). However, the desirability (probability of realizing the expected outcome) differs widely among the five cases. While the variance is assumed equal for C and D, the probability of getting a return equal to or greater than Z is obviously greater for C because it is skewed in the direction of higher rather than lower returns. Alternative B is preferable to A both because of (a) its smaller variance (range, standard deviation) and (b) its "flat peak" (kurtosis). While the mean expectation of X

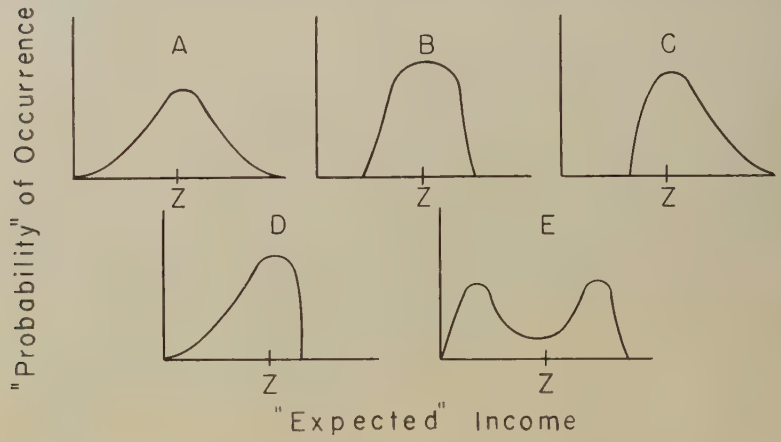

FIG. 6.—Hypothetical models of probability distribution illustrating different degrees of uncertainty.

income is as great under alternative E as under B, the firm's choice is largely an "either-or" one ("becoming wealthy" or "going broke") under the bimodal distribution.

QUANTITATIVE ESTIMATES OF VARIABILITY

The simple models above provide the relevant working hypotheses for estimating the effect of the combination of feed resources on uncertainty (variability) of returns. The data of Tables 2 and 3 (albeit crude) provide some notions here. [These relate to the variability (and distribution) of returns which grow out of changes in market prices for the finished commodities and the resources employed in their production.] We have not included physical variability in our calculations since (a) the problem under analysis is ordinarily posed in terms of market uncertainty and (b) data do not exist for study of income variability growing out of yield instability for the various crop and livestock producing systems. Thus in order to estimate the degree of market uncertainty we have followed this procedure in estimating the income for the various types of livestock and feeding systems for each of the years 1917–48: (a) The prices for each individual year have

been applied to the product produced (under the most nearly typical production and marketing system in each case). (b) The amount of resources other than feed (labor, building, and equipment depreciation, veterinary services, etc.) has been similarly estimated for each system and the costs in each of the individual years have been applied to all resources (feed, labor, interest, taxes, and all other expense items) for the given technique (in the sense that the resource inputs are held constant over time although variations between years in factor costs are recognized). Discrete forage-grain systems have been employed since presentation of the information for all combinations (e.g., each point on curve G_fF_f in Fig. 1) is impossible in limited space. Data have been assembled for the following groups of livestock. I. Hogs fed three levels of forage: (a) zero, (b) 10 per cent and (c) 20 per cent of the ration in dry lot. II. Hogs fed three levels of forage: (a) zero, (b) a daily grain ration equal to 3 per cent of the hog's weight, and (c) a daily ration equal to 1 per cent of the hog's weight on pasture. III. Choice feeder steers fed three levels of forage: (a) fed in dry lot, (b) fed on pasture, and (c) grazed on pasture and then fed out. IV. Dairy cows fed three combinations of grain: (a) the standard Haecker ration, (b) the medium forage ration, and (c) the highest forage ration in the Jensen study.[20]

If we assume a normal distribution and apply conventional tests of significance, no consistent statement can be made about variance of returns between feeding systems (within a given livestock system).[21] If dispersion of returns is taken as the measure of market risk or uncertainty involved, then the high-forage systems appear as safe as the high-grain systems within each livestock system.[22] Perahps a more note-

[20] Feeding systems are not actually closed in this sense because variations can be made even within a production period depending on price-cost relationships. However, in line with our stated objectives, we have included the systems enumerated as discrete cases. The data for feed inputs and livestock output have been taken from (1) the Beltsville experiment for dry-lot hogs, (2) Iowa experiment 101 for the pasture hogs, (3) Iowa project 1016 for feeder cattle and (4) the Jensen study for dairy cattle.

[21] The assumption of normality can, of course, be questioned outright. However, this problem likely has few implications for our conclusions.

[22] Bartlett's test of homogeneity of variance gives the following chi-square values for different systems within each type of livestock. (1) Returns above feed costs: (a) dry-lot hogs 2.7359, (b) pasture hogs 4.6504, (c) feeders .0358, (d) dairy cows .6405. (2) Return above all costs: (a) dry-lot hogs .3302, (b) pasture hogs .5871, (c) feeders .0712, (d) dairy coys 2.4326. (For two degrees of freedom, none of these values are acceptable at 10 per cent level.) These tests are between forage systems within each class of livestock and not between classes of livestock. If we use the standard deviation as an index (measurement) of net return variability, then we find a significant difference between classes of livestock at the 1 per cent level of probability. The relevant statistics are given below. The method employed without transformation of the variability observations and estimates of theoretical variance may over-estimate the variance not attributable to type of livestock and feeding systems. However, given the level of significance, these limitations appear unimportant:

Source of variance	*d.f.*	*s.s.*	*m.s.*	*F.*
Total	11	1032.0544		
Type of livestock	3	898.1336	299.3779	17.884
Feeding systems	8	133.9208	16.7401	

worthy comparison is that between types of livestock for parallel feeding systems (high-forage for hogs as compared to high-forage for feeder cattle, etc.). Were the goal one of utilizing forage in a manner which minimizes variance of income, the farmer should select dairy cattle first and feeder cattle last. The type of livestock is more important than the feeding system for a given class of livestock in minimizing income variability.

Examination of the frequency distribution in Table 4 as a rough indication of skewness and kurtosis (the shape of the distribution) also

TABLE 3

DISPERSION OF RETURNS FOR VARIOUS TYPES OF LIVESTOCK AND FEEDING SYSTEMS, 1917–48

Livestock and Feeding System	Return Above Feed Costs*		Return Above All Costs†	
	Standard Deviation	Coefficient of Variation Per Cent	Standard Deviation	Coefficient of Variation Per Cent
I Dry-lot hogs				
System a	43.36	23.37	21.54	20.03
System b	35.07	26.87	20.10	22.51
System c	32.26	29.44	19.38	24.81
II Pasture hogs				
System a	28.17	26.18	38.17	23.62
System b	56.95	31.66	26.48	24.03
System c	52.05	32.04	23.26	24.58
III Choice feeders				
System a	48.05	37.25	35.77	34.41
System b	47.56	34.72	34.43	32.80
System c	49.24	34.97	36.10	34.05
IV Dairy cows				
System a	49.72	24.13	10.79	11.02
System b	43.32	23.32	12.80	12.16
System c	49.05	23.89	14.48	13.17

* Feed only included as a cost in figuring returns.

† All cost items (including interest on investment, labor, taxes, power, equipment, etc.).

leads to contrasting conclusions. The chance of realizing more than $100 for an outlay of this amount (a profit above all costs including interest on investment) is consistently greater for hogs fed no forage than those fed the large amounts of forage. However, in the case of feeders and dairy cows, historic changes in price and cost relationships evidently have tipped the odds of positive profits in the direction of the higher forage rations. It is true, of course, that the profitability of the systems varies between years and hence that profits can be maximized were the operator to form "correct expectations" and adjust his operations accordingly (rather than consider the systems as "discrete" and follow any one without deviation over time). Yet these statements refer to the ("left" or "right") location of the entire frequency distribution (relative to a zero return). In terms of the conventional measure of skewness and kurtosis (the shape of the distribution irrespective of the

location), no outright statement can be made about the degree of uncertainty involved.

PHYSICAL RESEARCH

The analysis above has necessarily been limited to data of the kind and nature available. However, the implications of the derived coefficients are clearly evident. Additional data of the form presented should be obtained in the various state experiment stations if solution to feed production-utilization is to be provided on an extensive basis. While many of the crop rotation and animal feeding experiments provide basic data which are helpful in deriving the relevant structural rela-

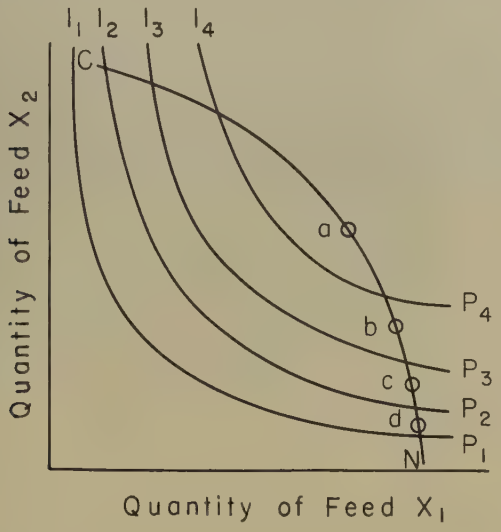

FIG. 7.—Hypothetical models of iso-quants illustrating different possible outcomes in experimental trials.

tionships, most are not so designed (because of limited funds and other considerations). While designed on an iso-quant (equal-quantity) basis, the given quantity is usually time or variables other than product. (The Beltsville, Jensen, and other experiments are exceptions.) Figure 7 can be used to contrast the nature of the conventional experimental design with that which might be employed were the objective one of deriving relationships of the nature outlined here. I_1P_1, I_2P_2, I_3P_3, and I_4P_4 are (conceptual notions or models of)iso-product curves (each representing a different level of output) such as those discussed previously and can be looked upon as several contours from a total family of contours on a production surface. However, the conventional design is more nearly expressed in a curve such as *CN* which also might be termed an iso-quant (equal-time, equal-capacity, equal-grade, etc.) curve. Hogs, for example, are fed a few different rations (points such

TABLE 4

FREQUENCY DISTRIBUTION OF RETURNS PER $100 OF ALL COSTS FOR SPECIFIED LIVESTOCK AND FEEDING SYSTEMS, 1917–48

Range of Returns per $100 of Costs*	Number of Years Estimated Income Fell in Specified Interval											
	Dry-lot Hogs			Pasture Hogs			Choice Feeders			Dairy Cows		
	a	b	c	a	b	c	a	b	c	a	b	c
Less than $100	12	25	27	12	17	22	29	17	16	20	13	9
$100–119	12	4	4	11	9	6	10	6	7	12	14	14
$120–139	5	3	1	5	3	2	5	3	2	0	5	9
$140–159	3	0	0	3	3	2	2	5	4	0	0	0
$160–179	0	0	0	1	0	0	5	0	2	0	0	0
$180–199	0	0	0	0	0	0	0	0	0	0	0	0
$200 and over	0	0	0	0	0	0	0	1	1	0	0	0

* A return of $100 per $100 cost means that all costs have been met and that the going wage rate for labor and interest on investment also would have been realized. Failure to receive $100 for each $100 in cost does not mean a farmer would have realized only losses. His income remaining after paying out of pocket costs could still be positive but would not necessarily have returned market prices on his labor, capital, and similar resources. The tests are between treatments within individual classes and not between classes of livestock.

as *a, b, c, d*) for an equal period of time. Analysis of variance is generally employed to test significance of differences between total or daily gains. Where the number of observations is small, estimates of substitution rates are impossible since these are confounded with transformation rates for a given ration. Even on the basis of designs of this nature, production functions and product contours can be derived from the resulting data if observations are great enough both for numbers of feed combinations and output levels (hog weights, level of milk production, etc.).

Animal husbandmen, agronomists, and production economists might well work cooperatively were the several structural relationships (which simultaneously are necessary for establishing the optimum combinations of farm resources) to be estimated. While many of the phenomena are of physical nature, models (basic logic) which are helpful in experimental design can also be drawn from production economics. Studies based on models of this nature would generally represent a departure from conventional experimental designs. For example, animal feeding experiments might be designed to provide estimates of a product contour or an entire production function (production surface). Experimental designs of this nature would imply (a) use of individual animals for estimating numerous points on a contour (such as I_1P_1, I_2P_2, etc. in Fig. 6) rather than replications of two or a few lots for a very few points (as *a*, *b*, *c*, and *d*) on a hybrid curve such as *CN* (which does not clearly establish either the feed substitution rates or the feed-product transformation coefficients) and (b) use of regression analysis to estimate the structural relationship (the entire feed contour or production surface) instead of analysis of variance to establish differences between means (a very few points on one hybrid curve).

SPRING DISPERSAL AND SETTLING ACTIVITIES OF CENTRAL IOWA MUSKRATS[1]

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Received April 18, 1951

This report presents the results of a field study of the spring dispersal and settling processes in the muskrat, *Ondatra zibethicus*. The study was planned to supplement work on spring and summer behavior of the species that Dr. Paul L. Errington of Iowa State College previously had been carrying on through marking and general observation. Many of the data were obtained during studies conducted from early spring of 1946 to midsummer of 1947 on approximately 14 square miles of Squaw Creek drainage extending northwest of Ames, Story County, Iowa (Fig. 1). Additional observations were made in the spring of 1947 on Big Wall Lake in Wright County, and Goose and Little Wall Lakes in Hamilton County, Iowa. These were all areas that had been kept under observation by Errington (1, 2).

TECHNIQUES AND CRITERIA

Overlapping field maps were made of the observed portions of the Squaw Creek area. The area was covered on foot each week and the locations, types, and relative quantities of muskrat sign (meaning any evidence of muskrat activity), weather, stream, and tracking conditions, and general remarks were recorded on the map of the sector concerned. Oxbow ponds, sloughs, pasture ponds, and contributing drainages in the immediate region were also investigated. The lower one-fourth of the main research area was chosen for particularly intensive study and was visited every other day or oftener when sign reading conditions were suitable. Lakes, drainage ditches, and small creeks included in the study area but located outside of the immediate Squaw Creek drainage system were visited at irregular intervals.

Tracks, fecal droppings of various ages, remains of food, repairs to lodges and burrows, disturbances of the creek bottom, and cloudy water in burrow entrances are types of sign looked for in following muskrat activities.

[1] Journal Paper No. J-1931 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 498. Fish and Wildlife Service (United States Department of the Interior), Iowa State College, Iowa State Conservation Commission, and Wildlife Management Institute, cooperating. The writer is indebted to Dr. Paul L. Errington for permission to use supporting data from field notes collected on the study areas in the years 1937–1945 inclusive. Weather records are those of the U.S.D.A. Weather Service Station on the Iowa State College Agronomy farm approximately 4 miles from the study area.

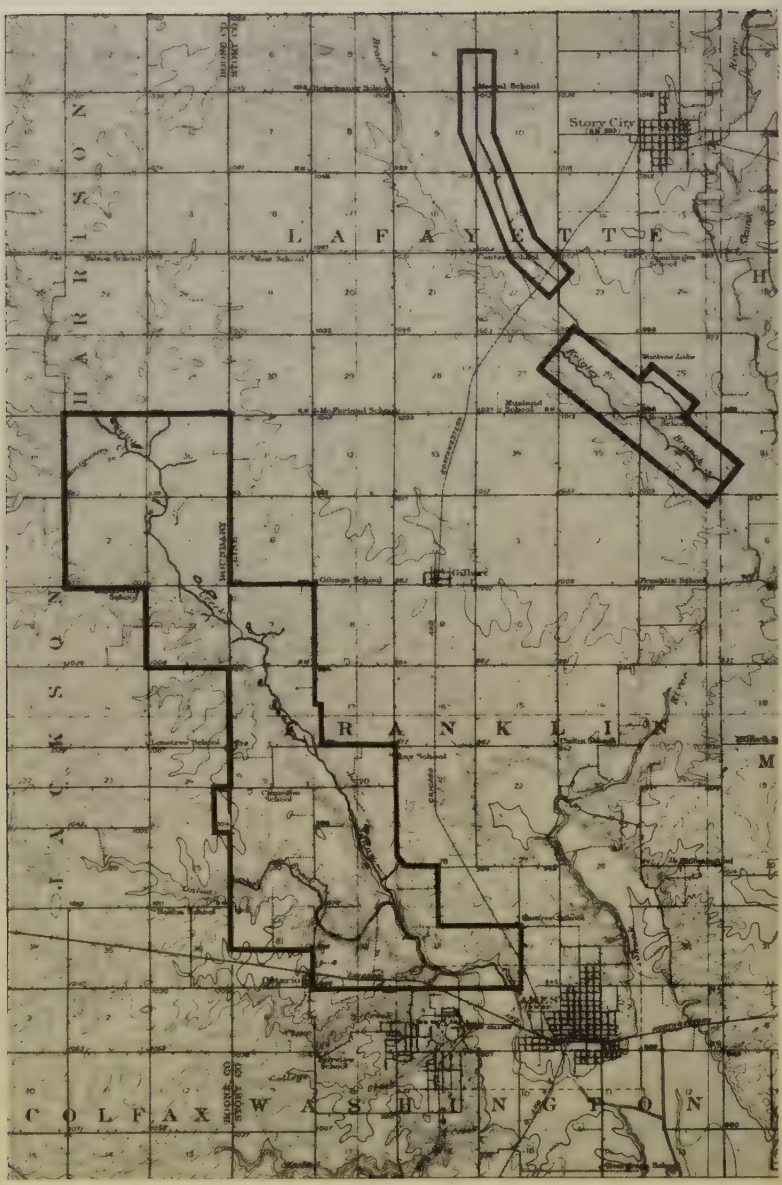


FIG. 1.—Map of the Story County, Iowa, drainage areas in this investigation.

The beginning of the spring dispersal was marked by relatively greater amounts and wider distribution of tracks and other sign. When general sign appeared along the streams and around the lake margins without reference to the limits of old territories the spring movement was well under way. Heavier mortality on roads and from predators suggested that the animals were in unfamiliar territory and probably were in the act of wandering or migrating. Sign appearing in many places where no muskrats were known to have wintered was conclusive evidence that dispersal was in progress. Predispersal restlessness was indicated by increased activity around the winter burrows and lodges but included no extensive movements.

It was possible to determine the sexual maturity of dispersing and settling muskrats by examining the victims of automobiles, disease, and predators. Approximate times of conception could be judged from the ages of the young found in territories.

Recognition of breeding territories was difficult early in the season when dispersal was still taking place. On the other hand, after dispersal had been under way for three to five weeks, the current sign was more concentrated and intense activity was confined to definite stream-bank areas of 150–250 yards in length. The sign in these areas was very abundant, of different ages, and generally included different types. If these small areas continued to show like sign until evidence of a litter of young were seen they were termed *breeding territories.* The territories could not be precisely delimited as, in most cases, their margins overlapped. A well used and extensive burrow system was considered to be strong evidence of a territory. Sometimes single transient animals set up quarters in relatively uninhabited locations but such settling was usually of an extremely temporary nature and the sites of activity could ordinarily be distinguished from true territories by the presence of less abundant sign. Breeding territories centering in lodges on the marshes were characterized by continued maintenance of the lodges and by lodge chambers containing young muskrats or lined with dry, well-chewed nesting materials.

THE INFLUENCE OF SOME AGENTS ON THE INITIATION AND RATE OF DISPERSAL

There seemed to be little relationship between the specific annual dates that spring dispersal commenced although the movement began progressively earlier each year in the period 1939 to 1943 and then later in the remaining years of the study (Table 1).

Floods were of little consequence in actually initiating the general spring movement. A predispersal flood in 1946 drove the animals to higher ground where they lived much like transients. When the high water receded the evicted muskrats gradually returned to their winter burrows. Similar responses were noted during predispersal flood conditions in 1939, 1943, and 1945. A flood occurring soon after movement had begun in 1945 appeared to drive the remaining animals from their wintering quarters and accelerated the migration.

Average air temperature, snow and ice conditions, degree of sexual maturity, and social intolerance together apparently have a bearing on the initiation and rate of spring dispersal, but no one of these factors alone was influential enough in itself to start the general movement. Markedly unfavorable weather tended to cause a slowing or temporary cessation of dispersal; this was twice noted to have occurred in 1943.

In the Squaw Creek area, average air temperature for three or more consecutive days seemed to be an important factor in initiating dispersal (Fig. 2). These averages were computed by averaging the even-hour temperatures of each day. For the three days preceding

TABLE 1
DATES OF INITIAL SPRING DISPERSAL BY SQUAW CREEK MUSKRATS

Year	Date Dispersal Began
1939	March 24
1940	March 18–19
1941	March 10–12
1942	March 6
1943	February 22
1944	February 25
1945	March 13–14
1946	March 16
1947	March 22

the initial movement in 1946, the average air temperature was 50°F., whereas in 1947 it was 32°F. In no one of the ten years for which data were available did dispersal begin until the air temperature averaged 32°F. or higher for the four days prior to and including the beginning date. Brief daily periods of fluctuations in temperature above and below 32°F. appeared to be of little immediate consequence. The effects of relatively warm periods in early 1939, 1942, 1943, 1946, and 1947 were offset by one or more adverse factors; in each instance, however, the muskrats exhibited restless activities in the vicinities of the burrows. Dispersal started slowly in 1942, 1946, and 1947 and gradually increased to full momentum. This gradual increase in migratory activity did not appear to be the direct result of extraordinary climatic conditions.

Snow and ice apparently have considerable effect on muskrat dispersal. During the period from mid-February to early March in 1946 moderately mild air temperatures prevailed and snow covered the ground although the streams were ice-free most of the time. On March 1 restlessness was noted in the vicinities of the burrows but activity decreased when more snow and colder weather came soon thereafter. In 1947 the streams remained frozen and snow was present during January, mid-February, and mid-March, although the weather was relatively mild. Dispersal began the day ice left the streams and sign of movement followed the retreating ice-cover upstream. During the

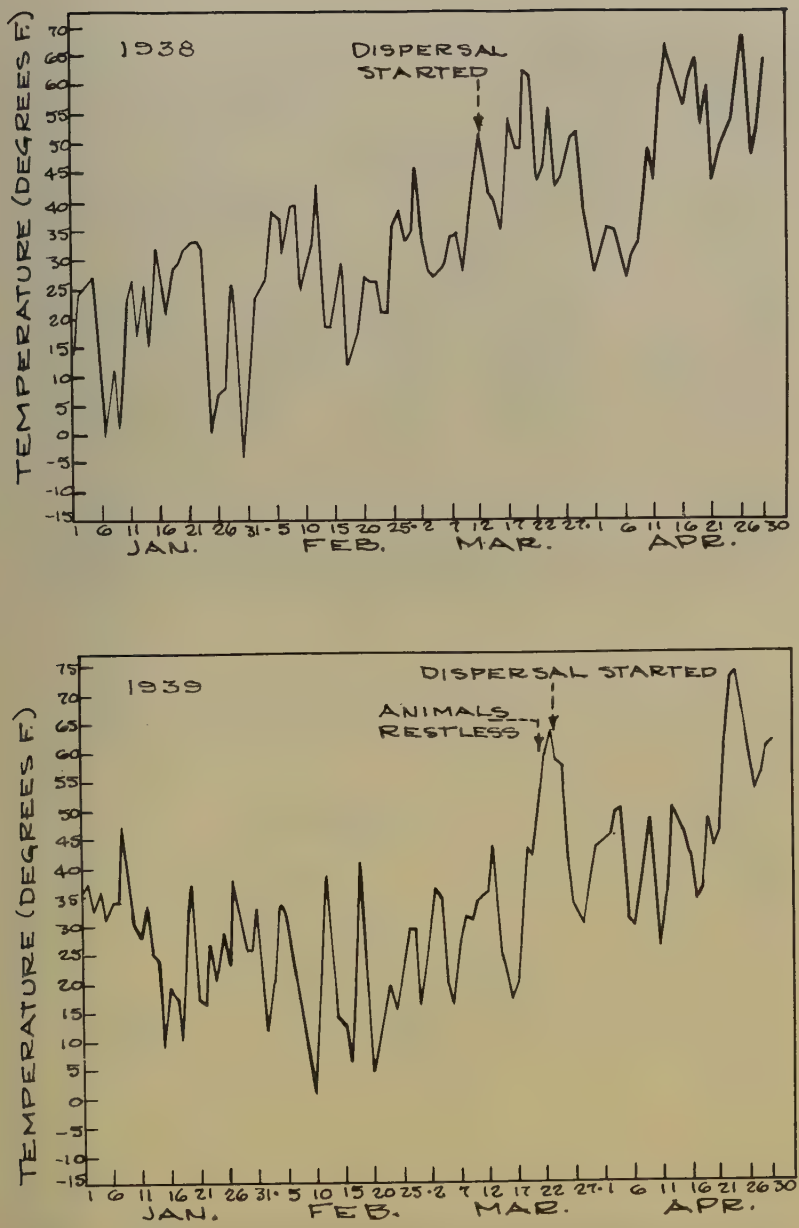
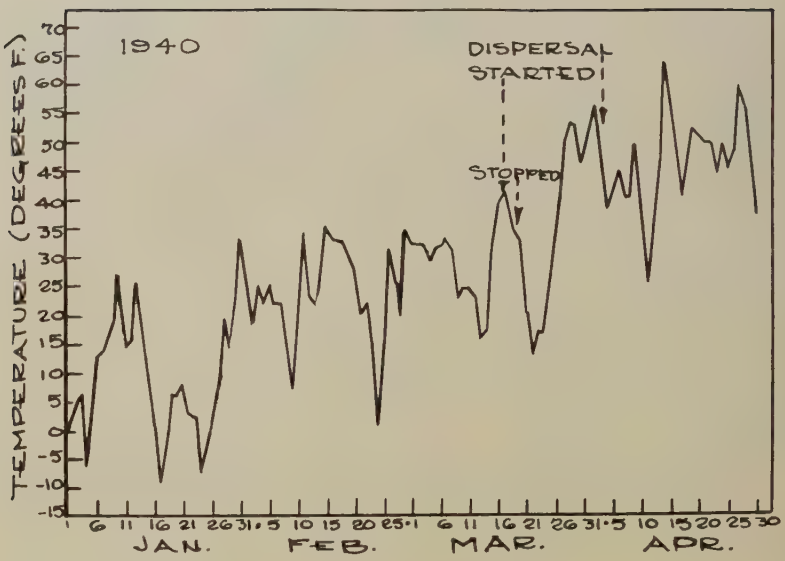
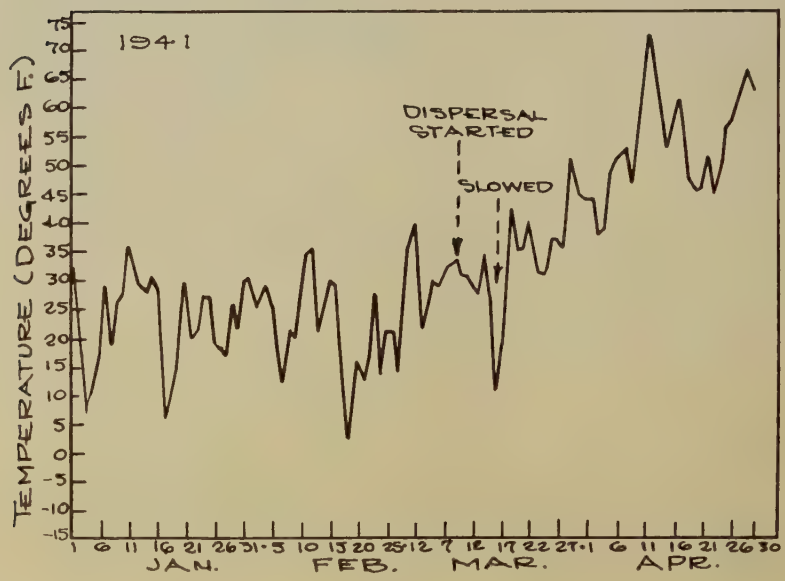


FIG. 2.—Average air temperatures for twenty-four hour periods beginning at noon of the dates indicated.

FIG. 2 (Continued)





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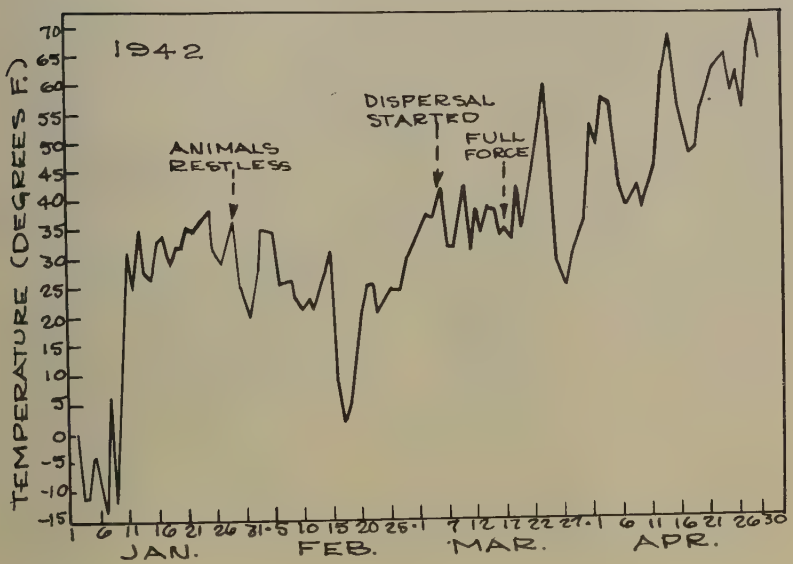

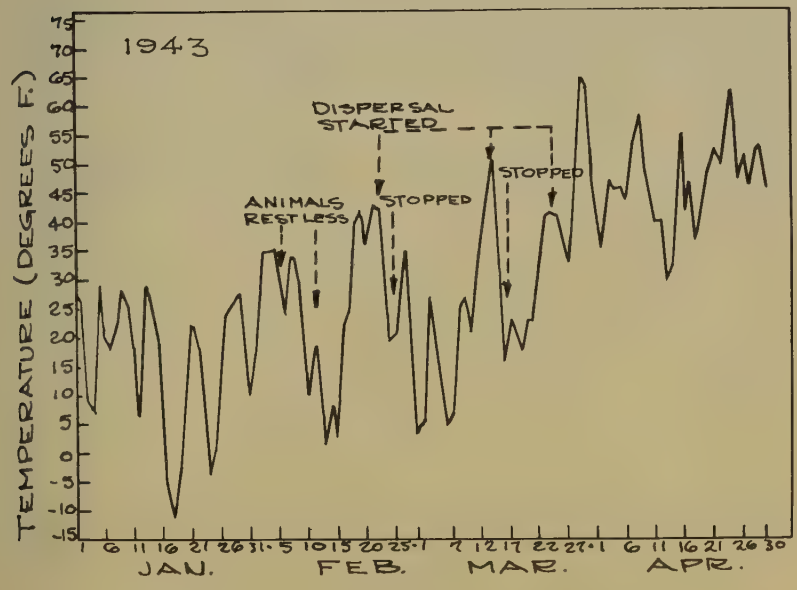

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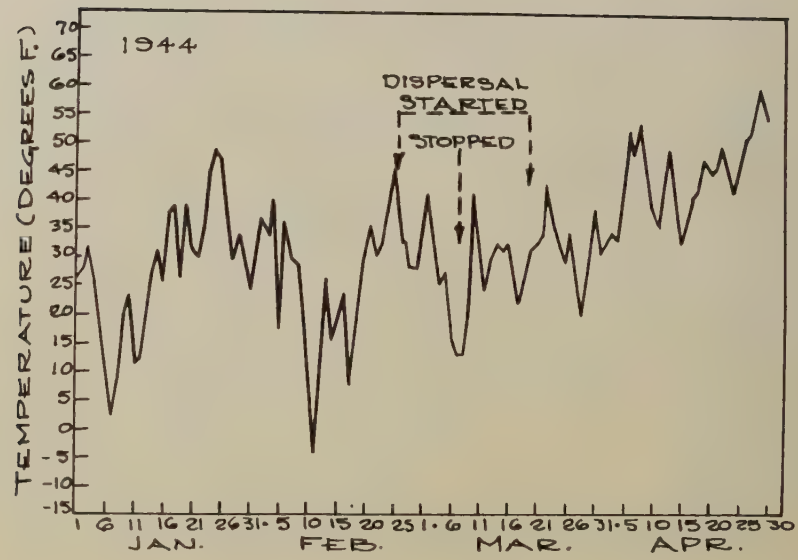

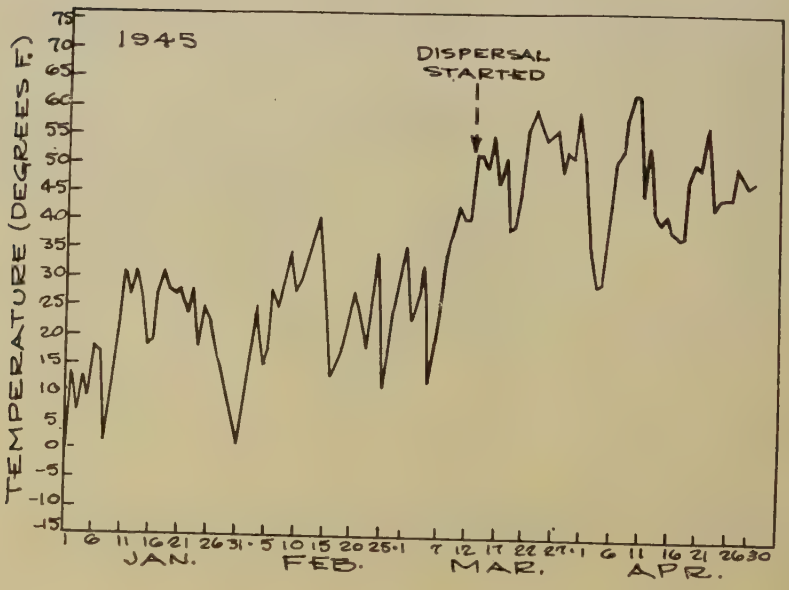

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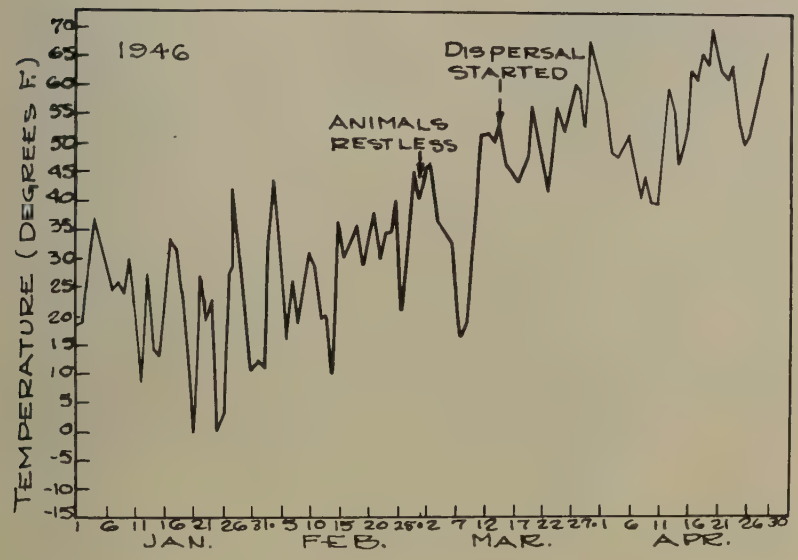

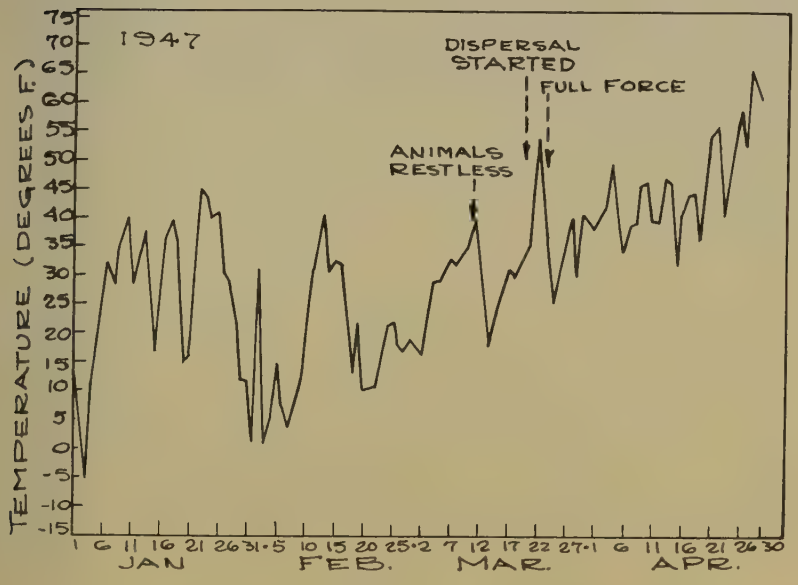

latter part of March snow melted rapidly (usually within the day following each storm) and caused little delay in the migration. In the years 1938 through 1943, inclusive, and in 1945 the streams were frozen and/or snow was on the ground during the predispersal periods. Though the streams were open, snow and colder temperatures slowed muskrat movement in mid-March, 1941 (Fig. 2). Similar conditions plus ice formation in the streams completely stopped dispersal in mid-March of 1943. In early March of 1944 snow and cold temperatures stopped movement even though the streams remained clear of ice. Partial ice coverings or large areas of drift ice seemingly delayed the dispersal on central Iowa lakes as much as ten days after it had begun in ice-free local streams in 1947.

As sexual awakening became advanced, signs of increasing restlessness were noticeable long before overland movement occurred [Errington, (2)]. The writer noted restlessness of this apparent nature in predispersal mild periods in 1946 and 1947 (Fig. 2). Similar periods of predispersal restlessness occurred in 1939, 1942, and 1943. Adverse winter food conditions may retard sexual development in muskrats and thereby delay the breeding season [Errington, (1)].

Examination of dead muskrats indicated that sexual development among the males was fairly well advanced by late January, 1947, in the central Iowa area but maturity obviously was not attained at the same time by all muskrats. In some cases breeding occurred before or during early dispersal. Muskrats one to four days old were found on May 7, 1947, in Big Wall Lake lodges although very little evidence of migration was noted on the marsh as late as April 11. Dispersal into Goose Lake began about April 16 and litters of young varying in age from one to twelve days were found on May 16. Although many female victims of an epizootic that were examined at Little Wall Lake shortly after movement began in 1947 were sexually mature animals, only a few showed evidence of coition.

Intraspecific friction other than that arising from sexual maturity probably influenced the early movement of some of the animals. Those suffering from crises, food shortages, overcrowding, and generally unfavorable living conditions were the ones tending to fight most among themselves [Errington, (1)].

ACTIVITIES DURING MOVEMENT

In its earliest stages spring dispersal seemed to be manifested chiefly by increasingly heavy activity in and around areas known to have wintered fair to good populations. Some animals would then work farther and farther away from the wintering sites. Following a nearly annihilative epizootic at Goose Lake, the first indications of repopulation during the 1947 dispersal were seen April 16 in the form of droppings and tracks in restricted areas along the south and southwest shores near the outlet; the newcomers later moved into and around the marsh.

A pronounced acceleration in dispersal activity was noted along Squaw Creek for two to three weeks after movement began in 1946 and 1947 and then the relative abundance of localized sign began to diminish. Increased numbers of transient shore-dwelling muskrats were present on Little Wall Lake nearly three weeks after dispersal began in 1947. A similar condition existed on Goose Lake as late as June 1 in 1943. These increases in numbers of transient animals living on shore probably resulted from increased intolerance among the deep marsh populations as breeding reached its peak or the first litters were born.

A number of breeding pairs continued to inhabit their wintering burrows throughout the summer months. Five such sites were recorded along Squaw Creek in 1946 where representatives of wintering groups remained between six and eight weeks after dispersal began.

The rates and distances traveled during the spring dispersal varied widely. Some animals moved only a few hundred yards before settling again; others, for miles. In a number of instances in 1946 and 1947 migration proceeded downstream but most dispersals took place upstream or across country away from the stream proper. Tracks of one animal were followed nearly a mile upstream on Onion Creek (tributary of Squaw Creek) March 21, 1947. On April 24, an animal was traced approximately one mile on Squaw Creek. Errington's notes record a large muskrat as traveling 1¾ miles in two days at the outset of dispersal in 1943. Droppings and tracks were found as far as 2,400 yards from any wintering sites on the date dispersal started in 1938, and three days later tracks were located several miles from the only places in which muskrats could possibly have wintered.

Temporary settling by transient individuals was of rather common occurrence. Lone wandering animals usually made "bachelor nests" in stream banks or along lake shores, and such quarters were occupied for varying lengths of time. In 1946 single animals came into the Squaw Creek area April 15 and May 9 and remained fifty-three days and nine days, respectively, before leaving. On April 19 two seemingly unassociated individuals established residences on a 300-yard stretch of Squaw Creek. One moved on after three weeks and the other after four weeks. In 1947 a pair arrived in a section of the study area eleven days after dispersal began and remained less than a week before moving on. A single animal moved into another stretch on or about April 9 and left again after three and one-half weeks. Still another remained ten days at one site.

Early in dispersal the stream animals ate exposed grass roots and made frequent visits to adjoining corn fields. There was evidence that they ate almost anything available. The color of fresh fecal droppings gradually became darker as a higher percentage of green material was added to the diet.

Muskrats in dispersal reacted in various ways to inclement weather and other crises. Errington (1) described the typical activities of migrating individuals caught by snowstorms and frigid temperatures as

well as the responses of the species to floods. In 1947 high water caused some dispersing and resident animals to leave Squaw Creek and go to higher ground or to quieter oxbow pools and bayous until the creek returned to normal levels. From all appearances many individuals frequenting these outlying retreats were hesitant about returning to the watercourses proper after having been driven out by high water. Many trips seemingly of an exploratory nature were made to the creek bank before the temporary quarters were abandoned.

Some unmated animals wandered erratically throughout the summer except for brief stops here and there. In 1946, one wanderer was recorded eighty-three days after the earliest movement. A male wanderer was collected ninety-two days after dispersal started in 1940 and an unbred female bearing strife wounds fifty-six days after dispersal started in 1943.

DURATION OF DISPERSAL PERIOD

Unless interrupted by cold weather or snow the dispersal periods in central Iowa were of relatively short duration. Many of the animals stayed in the wintering territories or nearby and a number of instances were found in which abandoned or trapped-out territories were occupied within a few days after movement started. By the end of a month and a half most breeding pairs were settled and dispersal activity had tapered off to that of vagrants and an occasional pair settling late. In 1946 several new pairs established territories on Squaw Creek soon after dispersal started but most of the remaining new territories were set up 27 to 54 days from the onset of general movement. Establishment of territories along the same watercourse was almost over by April 18, 1947, less than a month after dispersal began. Dispersal into Goose Lake began shortly before April 16, 1947. On this date the population was estimated to consist of the equivalents of five pairs that almost certainly had wintered there. Five more territories were recorded by April 23 and one territory per week was established thereafter until May 14 when the marsh population was judged to consist of the occupants of twelve definite breeding territories. Another pregnant female took up quarters on the latter date and a fourteenth territory appeared in late June.

PROCESS OF SETTLING

Typical of the exploratory activities exhibited in establishing a territory is a case noted in a hitherto uninhabited section of Squaw Creek on April 19, 1946. When first observed the sign was rather general over approximately 300–350 linear yards of stream bank but as the season progressed the radius of activity was gradually reduced to about one-half of that previously recorded and the quantity of sign increased correspondingly in the smaller area. Many tracks were noted along another extensive portion of Squaw Creek on April 18, 1947. Within a period of a week the sign had become consolidated and the smaller area was judged to constitute a territory.

A more complex procedure in settling was witnessed in 1947 in another part of the Squaw Creek drainage. A 200-foot ditch, which was normally dry, connected an old oxbow pond and the main stream. General and fairly abundant sign was found along the banks of the stream in this area when dispersal began but the activity was not of sufficient magnitude on March 24 to suggest a territory. Tracks and droppings of various ages had increased in abundance by April 3 to the extent that muskrats were believed to be settling on the main stream but the pasture ditch and the oxbow pond showed little evidence of current activity. Some changes in the location and consolidation of the sign were apparent by April 18 when the heaviest concentration of tracks was found to be closer to the mouth of the pasture ditch and evidence of exploratory wandering was noted in the ditch and about the oxbow pond. By April 29 many tracks and some droppings were still evident at the mouth of the ditch although the quantity was much reduced along the rest of the main stream bank. In the meanwhile the pasture ditch had become literally plastered with tracks leading to and from the oxbow pond and an old set of burrows in the pond had begun to show evidence of renovation and use. Very little sign was found on the stream bank and the ditch seemed little used on May 12 but the burrows in the pond were definitely occupied by a pair of muskrats. Tracks of young were observed in the vicinity of the burrows in early June.

A flattened lodge on Goose Lake showed first signs of repair on May 14, 1947, when a new portion containing a chamber large enough to accommodate one animal was added to the structure. Later on May 23, more remodeling had taken place but the size of the chamber remained about the same. A nest containing a litter of young of about eight days was found in the lodge on June 19. In this case the single female that had established the territory had been pregnant before she began to renovate the lodge.

Lone muskrats sometimes established territories and were joined later by animals presumably of the opposite sex. For example, an individual took up residence on a pasture pond west of Gilbert shortly before April 13, 1946, and lived alone until May 18 when another muskrat appeared and joined in establishing a breeding territory.

POST-SETTLING ACTIVITIES

After claiming a territory, the animals defended their "property" against trespassing muskrats. Fighting in these cases seemed to be more vicious than that noted in early dispersal.

Post-settling response to flood conditions was observed to a limited extent in the Squaw Creek area in 1947. Most of the adult animals and kits large enough to swim well successfully weathered the early June floods by going to higher ground or climbing on fallen tree stumps or debris. Heavy rains resulted in Squaw Creek being at high flood stage four different times in June of that year. Approximately one mile of the flooded valley was inspected by canoe on June 24; it was known to

have contained four definite territories with a possible fifth one being established just before the series of floods began. Although no animals were seen during the tour, the survivors were believed to have been hidden in the brush on the adjoining hillsides. At any rate, three territories were re-established after the flood waters had subsided.

Another portion of the watercourse was known to have had three typical territories and one single-animal territory prior to June 1. A post-flood examination June 28 revealed that the three breeding pairs had remained in the vicinity of their former territories but no sign of the single individual was evident. In two cases, the paired animals had not yet reoccupied their old burrows but were living temporarily in shallow overflow pools nearby, making many exploratory trips to the stream proper. Droppings of different ages were found on the trunk of a leaning tree in one of the pool areas, thus indicating that at least one muskrat had taken refuge there late in the flood period. The top of a fence post some 150 yards from the stream bed in the same area had a deposit of slightly older droppings.

Heavy rain early on June 30 resulted in a flash flood pouring through the Squaw Creek valley and some adult muskrats may have died. Two of three original territories were missing in a one-half mile portion of the creek on July 6 when a probably drowned female containing eight embryos was found lodged in driftwood. Many tracks between an oxbow and the site of one of the preflood territories indicated that one pair was reoccupying its former quarters at the time of the observation.

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JUVENILE RING-NECKED PHEASANT MORTALITY AND COVER UTILIZATION IN IOWA, 1949[1]

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Received May 2, 1951

Since 1935, with the exception of the period 1942–48, the Iowa Cooperative Wildlife Research Unit has been investigating the life history and management of the ring-necked pheasant (*Phasianus colchicus*) on the Winnebago Research Area. Although studies on the relationship of food and cover to winter survival [Green (3)] and nesting and production [Baskett (1)] have been made, only limited information on mortality and cover utilization by pheasant broods is available. Therefore, an intensive study into this phase of the life history of the pheasant was conducted from April to November, 1949.

Winnebago County lies within the Wisconsin drift soil area, which is characterized by a generally flat topography and few natural drainage channels. With the advent of intensive agriculture, most of the sloughs were drained by the laying of tiles and drainage ditches [Brown (2)]. Corn, oats, and soybeans compose the usual rotation with some fields being maintained in, or reverted to, hay pasture.

The Winnebago Research Area is in sections 9–12, 13–16, and 21–24, Eden Township, Winnebago County. The intensive brood study was restricted to sections 13, 14, 23, the east 320 acres of section 15, and the west 240 acres of section 24. In 1949, 93.2 per cent of the 2,480-acre area was under cultivation.

TECHNIQUES OF INVESTIGATION

Cover on the 2,480-acre area was checked twelve times from June 14 to October 21, 1949. During early morning (5:00–7:00 a.m.) observations on pheasant broods were made from an automobile by driving the roads surrounding the sections. The remainder of the day was devoted to field work, and an effort was made to proportion field time according to the total acreage of the six major cover types involved. A bird dog was utilized as an aid in locating broods and dead chicks.

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[1] Journal Paper No. J-1932 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 497. Fish and Wildlife Service (U. S. Department of Interior), Iowa State College, Iowa State Conservation Commission, and the Wildlife Management Institute cooperating.

[2] The writer is indebted to Dr. Theodore A. Bancroft, Iowa State College Statistical Laboratory, for guidance in the statistical analyses employed in this report and to Dr. G. O. Hendrickson, Dr. K. D. Carlander, and Dr. H. M. Harris, Department of Zoology and Entomology, Iowa State College, for their interest.

Brood data — such as location, number and age of chicks, time of day, temperature, wind, cloudiness, cover type with notations on height, density, and distance to cover edge — were recorded on a prepared form. If the observer believed that all of the chicks were counted, the brood observation was rated "good"; if not, "poor." A nest that contained one or more hatched eggs was considered as being successful.

For purposes of this investigation a brood was considered to be one or more chicks less than twelve weeks old accompanied by an adult pheasant. Broods of mixed ages were considered for cover utilization but not for size. After the birds were twelve weeks old, in some cases less, considerable mixing of broods was noted, and it was difficult to distinguish between juvenile and adult birds flushed in the field.

The age of broods was estimated by comparing their size to game-farm chicks of known age. Brood locations were plotted on a map, and an effort was made to avoid duplicating brood counts within a designated age group.

BROOD STUDIES

Of 192 broods observed, 81 were considered as good and non-duplicated counts. These furnished the data for brood mortality studies (Table 1).

The most difficult period of determining brood losses was when the broods were less than three weeks of age. Hens with broods were easily located by a bird dog, but difficulty was experienced in obtaining a good count of chicks. In most instances an accurate count was not possible. Therefore, an attempt was made to evaluate these losses by statistical analysis of the data obtained from successfully hatched nests and pheasant broods at three weeks of age.

Although other workers have found a relationship between clutch size and calendar dates, this study failed to reveal any such correlation for eleven successful nests. As a further check, the sixteen observed broods estimated to be three weeks of age were back-dated to their approximate biweekly hatching period, and a statistical comparison of these data with the biweekly hatching period of the eleven observed successful nests did not reveal any significant difference in date of hatching ($\chi^2 = 7.81$, .05 level $= 11.07$).

A statistical comparison of the mean number of hatched eggs in successful nests (brood size at hatching) and the mean brood sizes at three weeks of age did not indicate any significant differences ($t = 0.05$, .05 level $= 2.05$). The biological implication of these analyses is that the rate of mortality in pheasant broods from the time of hatching to three weeks of age was low. However, the mean of 7.0 hatched eggs in the eleven successful nests was less than the 8.7 found by Baskett (1) in 125 successful nests; therefore, the sample of successful nests might have been too small.

Inspection of the mean size of the pheasant broods in Table 1 indicates

a decline in numbers from the time of hatching through eleven weeks of age. A linear decline is significant as shown by $F = 4.90$, .05 level = 3.96. The largest drop (1.1 birds) was noted in the seven to eight weeks of age class. The 1949 mean for this group is comparable to pheasant brood data obtained in 1939, 1940, and 1941 by Baskett (1). Among broods more than four weeks of age none was observed with more than nine young.

Of the fourteen juvenile pheasants found dead, eleven were killed by tractor mowers — three were one week of age; seven, two; and one, eight. One, about six weeks of age, was killed by an automobile; and one, about two weeks of age, by a dog. The remains of a young pheasant were observed in a fox scat.

TABLE 1

FREQUENCY TABLE OF OBSERVED RING-NECKED PHEASANT BROODS, WINNEBAGO AREA, 1949

Brood Size (Number of Chicks)	Number of Observations					
	At Hatching	3 Weeks of Age	4 Weeks of Age	5–6 Weeks of Age	7–8 Weeks of Age	9–11 Weeks of Age
1		1				1
2					1	
3		1		1	2	1
4		1	1	2	4	2
5	3	3	1	3	2	2
6	2		1	3	1	6
7	3	1	3	1	2	1
8	1	4	5	7	3	2
9	0	3		3	2	2
10	1					
11	1					
12		2				
Total number of chicks	77	113	76	134	96	99
Number of brood observations	11	16	11	20	17	17
Mean number of chicks	7.0	7.1	6.9	6.7	5.6	5.8
Standard deviation	±2.00	±3.04	±1.38	±1.87	±2.26	±2.10

ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Among Groups	5	30.50	6.10
Linear effect	1	23.70	23.70
Deviation	4	6.80	1.70
Within Age Groups	86	416.40	4.84
Total	91	446.90	

TABLE 2

UTILIZATION OF COVER TYPES ON THE WINNEBAGO AREA BY RING-NECKED PHEASANT BROODS DURING VARIOUS PERIODS OF TIME, WINNEBAGO AREA, 1949

Cover Type	Acres	June 14–17	June 21–24	June 27 July 1	July 6–8	July 12–15	July 19–21	July 25–29	August 8–12	August 22–28	Aug. 31–Sept. 15	Sept. 20 Oct. 1	Oct. 12–21	Total
I. Hayfields	*227.7*	*0*	*2*	*2*	*7*	*4*	*12*	*9*	*12*	*12*	*5*	*0*	*0*	*65*
(9.2 per cent)			1		4	2	4		2	4	2			19
A. Alfalfa	69.1						1							3
B. Timothy	18.5		1	1										2
C. Native Grass (Meadows)	47.4							2						
D. Native Grass and Mixed Herbaceous (Road Ditches)	42.5				1	2	3	7	9	8	3			33
E. Canary Grass	10.4						1							1
F. Red Clover	13.2								1					1
G. Red Clover-Timothy	16.8			1	2		2							5
H. Alfalfa-Timothy	9.8						1							1
II. Small Grains	*659.7*	*0*	*0*	*2*	*4*	*13*	*11*	*10*	*1*	*2*	*3*	*0*	*0*	*46*
(26.6 per cent)				2	4	13	9	10	1	2	3			44
A. Oats	652.3						2							2
B. Wheat	4.8													0
C. Barley	2.6													
III. Oil Grains	*359.4*	*0*	*0*	*0*	*0*	*0*	*2*	*4*	*2*	*2*	*0*	*0*	*0*	*10*
(14.5 per cent)								1		2	0			3
A. Soybeans	241.6						2	3	2		0			7
B. Flax	117.8													

TABLE 2 (Continued)

Cover Type	Acres	June 14–17	June 21–24	June 27–July 1	July 6–8	July 12–15	July 19–21	July 25–29	August 8–12	August 22–28	Aug. 31–Sept. 15	Sept. 20 Oct. 1	Oct. 12–21	Total
IV. Pastures														
(7.6 per cent)	*188.0*	*0*	*1*	*0*	*0*	*2*	*4*	*7*	*3*	*0*	*6*	*0*	*0*	*23*
A. Bluegrass	29.4								1					1
B. Canary Grass	25.6													0
C. Native Grass	50.0						3	5	1		1			10
D. Red Clover-Timothy	50.8		1			1		1	1		4			8
E. Timothy	20.0						1							1
F. Timothy–Sweet Clover	12.2					1		1			1			3
V. Corn (35.3 per cent)	*875.3*	*0*	*0*	*2*	*1*	*0*	*7*	*2*	*6*	*8*	*2*	*0*	*0*	*28*
VI. Noncrop Land														
(6.8 per cent)	*169.9*	*0*	*1*	*2*	*1*	*0*	*2*	*5*	*3*	*5*	*1*	*0*	*0*	*20*
A. Fencerows	12.1			1			1	4	2	2	1			11
B. Sloughs	27.6		1	1						1				3
C. Roads and Lanes	22.4				1		1	1	1	1				5
D. Gravel Pit	1.6													0
E. Idle	8.2													0
F. Farm Groves and Lots	95.9									1				1
G. Schools and Churches	2.1													0
TOTAL	*2480.0*	*0*	*4*	*8*	*13*	*19*	*38*	*37*	*27*	*29*	*17*	*0*	*0*	*192*

COVER UTILIZATION

One of the objectives of the study was to obtain a better comprehension of the type of cover utilized by pheasants of different ages and at various periods of the summer. A total of 192 pheasant broods was observed in the various cover types on the 2,480-acre area (Table 2).

On the basis of adjusted field acreages, hayfields were used to the greatest extent, followed by pastures, noncultivated land, small grains, corn, and oil grains respectively. The latter two cover types were not used extensively until after the oats were harvested, about July 19.

Table 3 presents a statistical analysis of the data in Table 2 be-

TABLE 3

AN ANALYSIS OF VARIANCE OF THE UTILIZATION OF COVER TYPES BY PHEASANT BROODS DURING VARIOUS PERIODS OF TIME (TABLE 2), WINNEBAGO AREA, 1949

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	*F* Values
Cover type (*C*)	5	50.504	10.101	11.261
Periods (*P*)	8	22.798	2.850	3.177
Linear	1	6.847	6.847	7.633
Quadratic	1	4.295	4.295	4.788
Cubic	1	0.662	0.662	0.738
Residual	5	10.994	2.199	.....
C x *P* (error)	40	35.889	0.897	.....
Total	53	109.191	.....	.....

tween June 21 and September 15, inclusive. Assuming that cover type times period ($C \times P$) interaction is a valid estimate of error, tests made in the next paragraph are appropriate. Further, cover times period ($C \times P$) is only considered to be a valid estimate of error if the periods were selected at random. Although the selection of periods was not strictly at random, the periods cover the season in which one would expect to find pheasant broods from one to eleven weeks of age. Also, the nine observational periods were scattered at approximately equal intervals throughout the season.

There is a statistically significant difference ($F = 11.26$, .01 level $= 3.51$) in brood utilization of the various cover types and in the number of broods observed during the nine periods ($F = 3.18$, .01 level $= 2.99$). The statistical components of periods (Table 3) indicate that the number of observed broods increased in a linear fashion as the summer progressed; however, if the analysis had been continued for a period of several more weeks, the quadratic regression probably would have

been significant as the number of broods less than twelve weeks of age would be decreasing.

Table 4 presents the relationship of pheasant broods in various age classes and the utilization of major cover types. A decline in the utilization of small grains by pheasant broods more than six weeks old is related to the harvest of these grains.

An analysis was made of the utilization of various densities of cover type by ring-necked pheasant broods. The densities of the various cover types, excluding corn, were determined by visual estimate at the site from which the brood was flushed. If less than 50 per cent of the ground litter was covered by vegetation, the cover was considered to be sparse; 50 to 90 per cent, medium; and more than 90 per cent, dense.

TABLE 4

RELATIONSHIP OF COVER UTILIZATION AND RING-NECKED PHEASANT BROODS, WINNEBAGO AREA, JUNE 14–OCTOBER 21, 1949

Cover Type	Age in Weeks					Total Number Broods
	1–2	3–4	5–6	7–8	9–11	
I. Hayfields	10	18	11	13	13	65
II. Small grains	12	13	12	7	2	46
III. Oil grains	1	2	3	2	2	10
IV. Pastures	2	6	5	4	6	23
V. Corn	3	5	3	8	9	28
VI. Noncultivated land	2	3	6	4	5	20
Total	30	47	40	38	37	192

Table 5 presents the relationship of cover types and density to brood utilization. As a rule, the majority of broods were found in vegetation of medium density. Statistical analysis indicated a significant difference in the utilization of various cover types at the three density levels by pheasant broods ($\chi^2 = 19.98$, .05 level = 15.51). No measure is available as to the relative abundance of the three density classes in the different cover types.

The utilization of various cover types at different heights is presented in Table 6. Corn was not used to any noticeable extent until it was sixty or more inches in height. The increased utilization of corn was closely related with the harvest of small grains.

PRODUCTION

All adult females observed while driving the section-line roads between 5:00 and 7:00 a.m. during August and September, 1949, were recorded, and notations were made on the number of young birds with

TABLE 5

Relationship of Cover Types and Density to Utilization by Ring-Necked Pheasant Broods, Winnebago Area, June 14–October 21, 1949

Cover Types	Broods Observed According to Cover Density			Total Number of Broods
	Sparse (More than 50 Per Cent of the Soil Litter Visible)	Medium (50–90 Per Cent of the Soil Litter Covered)	Dense (Less than 10 Per Cent of the Soil Litter Visible	
Hayfields	8	31	26	65
Small grains	10	32	4	46
Oil grains	3	5	2	10
Pastures	6	9	8	23
Corn				0
Noncultivated crops	6	6	8	20
Total	33	83	48	164

them. A total of 56 females and 219 young were observed for a ratio of 3.9 young per female. Of the 56 females, 45 (80.3 per cent) were with young birds.

These data may be of some value on a comparative basis from year to year and should not be interpreted as representing the number of females that produced young for two reasons. Field evidence indicates a decided difference in behavior of adult females with and without young, and nonproductive females may be associated with pheasant broods.

TABLE 6

Number of Broods Observed in Cover of Various Types and Heights, June 14–October 21, 1949

Cover Type	Height of Cover in Inches							Total Number of Broods
	0–5	6–11	12–23	24–35	36–47	48–59	60+	
Hayfields	9	11	32	13				65
Small grains	8	16	6	13	3			46
Oil grains	1	2	2	3	2			10
Pastures	8	7	6	2				23
Corn					3	2	23	28
Noncultivated land	5	5	7	3				20
Total	31	41	53	34	8	2	23	192

SUMMARY

1. The report covers observations on ring-necked pheasant broods during the summer of 1949 on a 2,480-acre tract on the Winnebago Pheasant Research Area.

2. Of the 192 broods observed, only 81 were considered to be "good" and nonduplicated counts within an age period.

3. Statistical analyses of means from successful nests and broods at three weeks of age did not reveal a significant rate of mortality.

4. A significant linear regression in pheasant brood sizes was found from one to eleven weeks of age.

5. Mowing of hayfields accounted for the greatest observed loss of juvenile pheasants.

6. On the basis of adjusted field acreages, hayfields were the cover type utilized to the greatest extent by pheasant broods. Following in order of preference were pastures, noncultivated land, small grains, corn, and oil grains.

7. Fields of corn and oil grains were not used extensively by pheasants until after small grains were harvested.

8. Statistical analyses of the utilization of three degrees of cover density in various cover types varied significantly. Cover with a medium density was frequented more than light or dense cover.

9. Roadside data during the months of August and September, 1949, indicated 3.9 young per female, and 80.3 per cent of the females were with young.

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"REDUCED" VASCULAR BUNDLES IN MAIZE[1]

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Received June 8, 1951

The mature vascular bundle of maize consists essentially of the phloem, the tracheal and parenchymatous elements of the xylem, and the encasing sheath of sclerenchyma. In the bundles near the center of the stem, the bundle sheath consists of a few layers of relatively thin-walled cells. The phloem occupies a large sectional area of the bundle. The protoxylem consists of three to five annular or spiral vessels, (= tracheae or tracheal tubes), arranged in a radial row and flanked by considerable xylem parenchyma. The metaxylem consists of two large porous-end pitted vessels, and a group of reticulate-end pitted tracheal elements. The typical bundle is described by Hayward (2).

Toward the periphery of the stem, a progressive change in the cellular composition of the bundles is evident. The sheath becomes progressively wider and its cells thicker-walled and more lignified. The phloem comprises less of the sectional area. The protoxylem elements and xylem parenchyma cells are fewer in number or absent. In the metaxylem, the pitted-end tracheae are also fewer in number or absent, and only one porous vessel may be present. Thus, a vascular bundle may be "reduced" to a single trachea, encased in a wide sclerenchymatous sheath.

In addition to this range of bundle structure within the stem of maize, some lines and varieties have distinctive features of bundle structure and bundle distribution. A striking bundle character, the reduction of some bundles to a strand of sclerenchyma, has been observed in several inbred lines and hybrids of maize. The present description of this character is a minor phase of the study of the histology of the maize stem in relation to mechanical strength, and in relation to fungal and insect invasion.

MATERIALS AND METHODS

The plants used in this study were obtained from the plantings of the Agronomy Department through the courtesy of Dr. G. F. Sprague. The numbers of the lines are given in connection with the observations. Samples were taken from the first internode above the highest anchored

[1] Contribution from the Botany and Plant Pathology Department and the Agronomy Department (Farm Crops), Iowa Agricultural Experiment Station, Ames, Iowa. Journal Paper No. J-1874 of the Iowa Agricultural Experiment Station, Project 1201.

brace roots. This internode is not the same number of nodes above the kernel in all plants, but it is the internode where stresses are comparable. No systematic scheme was devised for selecting plants because this aspect of the study is entirely descriptive, and no quantitative implications are intended.

Transverse disks were cut from the stem with a fine-toothed power saw and killed and stored in FAA. The disks were subsequently subdivided and trimmed with a razor blade to remove bruised tissues. The remaining sectors, which included only the outer fourth of the stem, were embedded in paraffin by the dioxan-normal butyl alcohol or dioxan-tertiary butyl alcohol method. All of the lines and varieties studied, except one South American variety, were easily sectioned in paraffin. The sections were stained in hemalum-safranin or safranin-fast green.

OBSERVATIONS

The inbred lines of maize L289 and I205 and the hybrid L289 $\times$ I205, conform to the pattern of distribution and structure of vascular bundles characteristic of maize (Fig. 1). A complete bundle that has all of the possible kinds of vascular and nonvascular elements is shown in Fig. 2. A somewhat reduced bundle that lacks protoxylem is shown in Fig. 3. Reduction of the metaxylem to a single trachea is also common in peripheral bundles.

The climax of reduction in the above lines is found in peripheral bundles that have no vascular or parenchymatous elements. Such strands consist entirely of sclerenchyma (Fig. 4). The smallest reduced strands may have less than ten cells in section, whereas the largest strands approach the sectional area of normal vascular bundles. The strands occur at random around the periphery of the stem. The proportion of reduced bundles is too erratic in samples of the above lines to justify quantitative study. Their vertical extent throughout the stem has not been studied, but it has been noted that within an internode, the sclerenchyma strands anastomose with the inner portions of the sheaths of normal bundles.

The character has appeared consistently, but in varying degree, for three successive years in field-grown plants of the above lines, but it has failed to appear in greenhouse-grown plants. Reduced bundles have been found in inbred lines other than the ones described here. A survey of the incidence of the character in inbred lines and hybrids is being continued in conjunction with other studies of stem structure.

DISCUSSION

The fact that the highly reduced vascular bundles described in this report have not attracted wider attention suggests that they may be associated with the highly specialized nature of inbred lines. This suggestion could be tested by a survey of open pollinated strains, which have almost disappeared from the Corn Belt, but are still grown extensively in other areas.

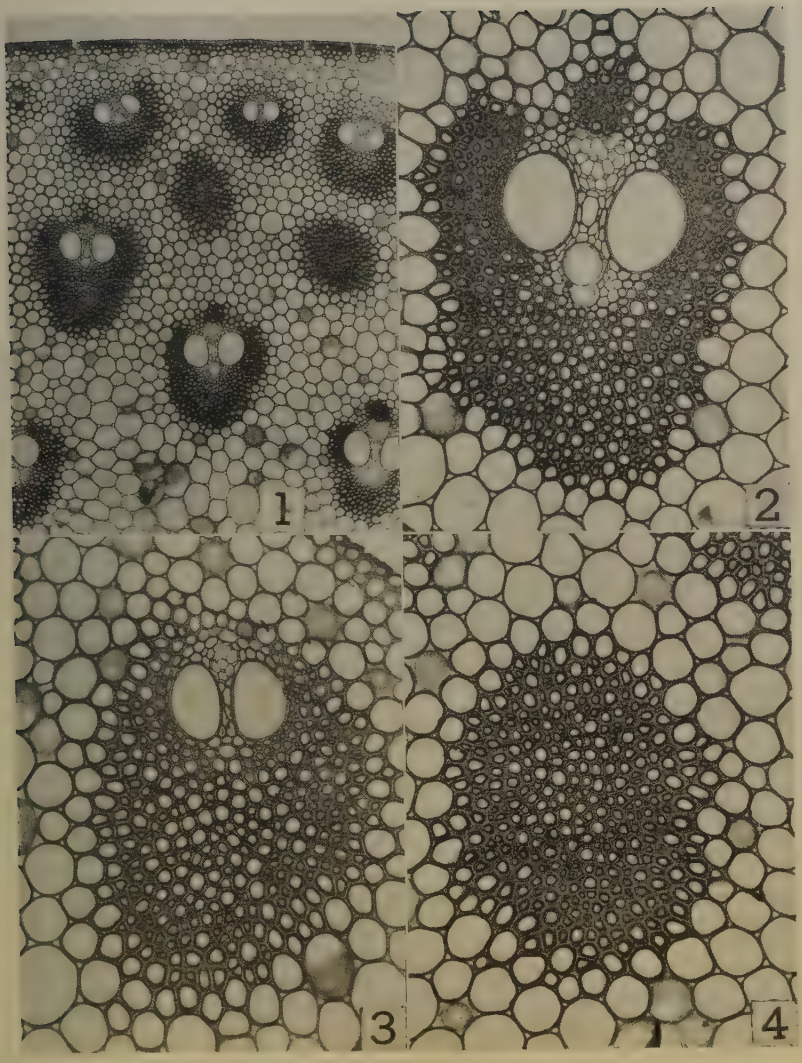

Fig. 1.—Cross section of peripheral region of the stem of Zea, hybrid L289 x I205. 45x.

Fig. 2.—Complete vascular bundle from sub-hypodermal zone, L289 x I205. 140x.

Fig. 3.—Somewhat "reduced" bundle from region near the hypodermis. Note the lack of protoxylem. L289 x I205. 140x.

Fig. 4.—Highly "reduced" bundle, lacking vascular elements. L289 x I205. 140x.

A study of the ontogeny of the reduced bundles is desirable to augment Miss Esau's critical description of the ontogeny of the maize bundle (1), and Sharman's description of the vascular pattern (3), and to add information concerning the derivation of the bundle sheath in relation to the derivation of xylem and phloem. If a line of maize having a high incidence of completely reduced bundles can be found, especially if the character can be consistently obtained in the greenhouse, an ontogenetic study can be made more effectively than with the lines studied to date.

Possible phylogenetic implication of this character should be approached by further search for the character in *Tripsacum and Euchlaena*, in which the character has not been observed.

SUMMARY

The stems of several inbred lines of maize, and their hybrids, have some vascular bundles that lack xylem and phloem, and consist exclusively of sclerenchyma.

These "reduced" bundles have recurred consistently in the field during three seasons, but have not occurred in crowded, potted plants grown in the greenhouse.

Because of the virtual disappearance of open pollinated maize from the Corn Belt, a search for the above character in open pollinated strains is proposed in regions where such strains are still common.

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THE VALUE OF THE DEGREE-HOUR SUMMATION SYSTEM FOR ESTIMATING PLANTING SCHEDULES AND HARVEST DATES WITH SWEET CORN IN IOWA [1]

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Received June 8, 1951

The development of a planting schedule is perhaps the most important phase of field work for canners. A systematic method is necessary to offset seasonal variations during the planting season and, projected further, to prevent gluts of corn in the processing plant at harvest time. Regular intervals used in planting sweet corn, the number of days from planting to harvest, and the period from silking to harvest are criteria that have been utilized in developing planting schedules. None of these has proved satisfactory. The system of cumulative degree hours, based on temperatures above an established base involving a summation of daily degree hours, has been correlated with the growth and development of several canning crops, particularly sweet corn and peas. This system has been used to schedule plantings and to predict harvests.

The studies reported in this paper present information on the application of the cumulative system of degree hours obtained from data on planting and harvest dates for a number of years for several hybrid sweet corn strains at Ames, Iowa.

The term *heat units* is a misnomer when the nomenclature of physics or meteorology is considered. The term, *heat units,* has been in general use in the literature and in the canning trade for a considerable time, and any change in terminology deviating too greatly would be confusing to workers in the field. However, in this paper the term *degree hours* will be used as synonymous with the term *heat units.* The term *degree hours* will be used both on a daily and cumulative basis.

LITERATURE REVIEW

Data in the literature relating to the effect of temperature on the development and maturity of crop plants are voluminous. The study of cumulative temperature effects on crops, particularly as related to prediction of date of blossoming or date of harvest for the commercial grower, is interesting from the application standpoint. Appleman and Eaton (1) and Magoon and Culpepper (9, 10) found that temperature was an important factor in determining the length of the maturity period. Culpepper and Magoon (7) stated that rainfall and other factors besides

[1] Journal Paper No. J-1948 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1186.

temperature also influenced the length of time between planting and maturity.

In establishing a base for growth in temperature Livingston and Livingston (8) used the figure of 40°F. In subsequent work with sweet corn, Magoon and Culpepper (10) used the figure of 50°F. for a base. Scott (12) stated that the minimum growing temperature for peas is approximately 40°F. and for corn 50°F.

Boswell (4, 5), working with peas, found that in successive plantings the later planting required less time to maturity than the preceding one and the higher the temperature the less time required for a given period of development. Magoon and Culpepper (10) stated that corn subjected to drouth conditions showed a developmental response to prevailing temperatures that differed from the response of the same variety which had ample rainfall. More heat seemed to be needed to bring the corn subjected to drouth to maturity.

Barnard (2) explained the application of heat units as used by the Green Giant Company (Minnesota Valley Canning Company). He stated:

> The eight fundamentals which seem to govern the whole theory are:
>
> (1) The daily mean temperature varies over any given period from year to year. These variations are indicated as departures from the normal temperature for a given date.
>
> (2) Each crop has what might be termed a *critical temperature* below which it will not grow.
>
> (3) Each crop will make growth toward maturity at a rate which is in direct proportion to the temperature.
>
> (4) Each crop is hurt by temperatures above that point at which it makes its best growth. (It is well known, for example, that corn will stand at least 10 degrees more heat than peas.)
>
> (5) Each crop, and each variety of a crop, has what we have termed a *total heat unit requirement* which will not vary, other factors being equal.
>
> (6) There are several factors which we have found to affect the use of temperature by the growing plant, including:
>
> a. Soil fertility — low fertility results in slower growth.
> b. Soil type and topography — light soil and a south slope result in faster growth.
> c. Depth of planting.
> d. Soil drainage.
> e. Vigor of seed.
> f. Cultural practices — root cutting in cultivation slows growth of corn. There are other factors which may or may not affect the temperature — maturity relationship, for example. Drouth may hasten maturity.
>
> (7) There must be as many heat units intervening between each planting as will occur under normal conditions at harvest.
>
> (8) Allowance must be made in a planting schedule for the factors other than temperature which affect maturity.

Bomaloski (3) Phillips (11) and the Crops Consultants of the Continental Can Company (6) were in agreement with the above fundamentals.

Bomaloski (3) found a good correlation between the accumulation of *growing degree days* (1/24 of degree hour summation) and the tenderometer reading increase for peas. He gave tables showing the extensive

variation of growing degree days of each season as compared with the average. Phillips (11) presented data on pea and sweet corn varieties giving the range in heat units that could be expected when several crop seasons were considered. He stated "the heat unit theory is excellent for planning a planting schedule that will give even flow of acres to be harvested during each day of the canning season and avoid bunching to a large extent."

The Crop Consultants' Memorandum of the Continental Can Company (6) gave a detailed report on the use of the heat unit system based on degree hours and the application of the heat unit theory to peas and corn on a commercial basis.

MATERIALS AND METHOD

Dates of planting and harvest were available from the sweet corn hybrid yield tests from 1938 through 1950 for three golden hybrids, Goldencross, Ioana, and Tendermost; four white hybrids, Silvercross, Illinois 14 x 11, Iogreen 56, and Illinois 14 x 13; and four Country Gentlemen types, Iogent 11, Iogent 27, Illinois 8 x 6, and Iogent 12. The minimum number of years any hybrid was grown was 5 and the maximum was 12. The daily maximum and minimum temperatures for each of the above seasons were obtained, and the total number of degree hours for the growing season from the date of planting to, but not including, the date of harvest were accumulated for each season. The formula utilized (2) for the accumulation of the daily degree hours was:

$$\Sigma\left[\left(\frac{\text{maximum temp.} + \text{minimum temp.}}{2} - 50\right)24\right] \text{ where}$$

Σ = summation
50 = the base temperature for minimum corn growth
24 = hours per day

Also, from another experiment with sweet corn, information on dates of planting was available for the years 1937, 1938, and 1939. The planting dates were at weekly intervals beginning May 1 and continuing for 7 weeks to approximately June 13. The cumulative degree hours were obtained for each weekly planting for each hybrid. The hybrids grown were Goldencross, Iogent 12, and Evergreen 191 x 1248.

All the material was grown at the Horticulture Farm, Iowa State College, Ames, Iowa, on a fertile Webster-type soil. The daily temperature data for all the seasons was collected from the Agronomy Farm records by the Agronomy Department (Climatology). This farm is located approximately two miles south of the Horticulture Farm.

The sweet corn was harvested at the optimum canning stage for all types of hybrids.

EXPERIMENTAL RESULTS

From the actual number of degree hours for each season for each hybrid, an average number of cumulative degree hours can be obtained

TABLE 1

DATE OF PLANTING, ACTUAL AND AVERAGE NUMBER OF DEGREE HOURS, AND THE ACTUAL AND PREDICTED DATE OF HARVEST AND DAYS TO HARVEST FOR THE GOLDEN HYBRIDS

Date of Planting		Tendermost					Ioana					Goldencross				
			Actual		Predicted			Actual		Predicted			Actual		Predicted	
		Degree Hours	Hvst Date	Days to Hvst	Hvst Date	Days to Hvst	Degree Hours	Hvst Date	Days to Hvst	Hvst Date	Days to Hvst	Degree Hours	Hvst Date	Days to Hvst	Hvst Date	Days to Hvst
1950...	5/31	38724	8/25	86	9/6	98	38100	8/24	85	9/5	97	38724	8/25	86	9/2	94
1949...	5/27	44424	8/12	77	8/10	75	44424	8/12	77	8/9	74	43692	8/11	76	8/8	73
1948...	5/27	43248	8/21	86	8/21	86	42516	8/20	85	8/21	86	41976	8/19	84	8/19	84
1947...	6/4	47856	8/29	86	8/20	77	47148	8/28	85	8/20	77	46080	8/26	83	8/19	76
1946...	5/24	45372	8/24	92	8/19	87	45372	8/24	92	8/27	95	45000	8/23	91	8/16	84
1945...	5/24	40368	8/22	90	8/28	96	45036	8/31	99	8/19	87	40368	8/22	90	8/26	94
1944...							44712	8/16	80	8/12	76	43092	8/13	77	8/11	75
1943...	5/28						40272	8/8	81	8/13	86					
1942...	5/19						42336	8/14	77	8/14	77	41952	8/13	76	8/14	76
1941...	5/29						42372	8/15	78	8/16	79	40656	8/12	75	8/14	77
1940...	5/29						39312	8/13	75	8/17	79	39660	8/11	73	8/15	77
1939...	5/30						43666	8/10	80	8/8	78	42864	8/9	79	8/7	77
1938...	5/22															
Aver. degree hours		43320					42939					42188				

for each hybrid. This average will be an indication of the maturity period of the hybrid based on daily degree hours. Similar values may be obtained in degree days (3) or in actual days from planting to harvest.

Tables 1, 2, and 3 present for each hybrid the average number of degree hours, the actual yearly degree hours, the actual date of harvest and days to harvest, and the predicted date of harvest and days to harvest. The predicted date of harvest and days to harvest are based on the average degree hours for each hybrid.

The predicted harvest date and days to harvest for each hybrid are obtained by projecting the average degree hours required to the date of harvest in the normal temperature curve. The day upon which the cumulative degree hours from the normal temperature curve coincides with the average cumulative degree hours required to the date of harvest will be the predicted day of harvest. Table 1 presents data for the golden

TABLE 2

DATE OF PLANTING, ACTUAL AND AVERAGE NUMBER OF DEGREE HOURS, AND THE ACTUAL AND PREDICTED DATE OF HARVEST AND DAYS TO HARVEST FOR THE WHITE HYBRIDS

Date of Planting		Degree Hours	Actual		Predicted		Degree Hours	Actual		Predicted	
			Hvst Date	Days to Hvst	Hvst Date	Days to Hvst		Hvst Date	Days to Hvst	Hvst Date	Days to Hvst
		Silvercross					Ill. 14 x 11				
1950	5/31	39084	8/26	87	9/3	95	.....	.....	.....	.....	.....
1949	5/27	44424	8/12	77	8/8	73	46980	8/16	81	8/16	81
1948	5/27	42516	8/20	85	8/20	85	46332	8/25	90	8/26	91
1947	6/4	47856	8/29	86	8/20	77	52645	9/4	92	8/27	84
1946	5/24	45372	8/24	92	8/19	87	.....	.....	.....	.....	.....
1945	5/24	40368	8/22	90	8/26	94	45804	9/1	100	9/3	102
1944	6/2	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1943	5/28	43860	8/14	78	8/11	75	.....	.....	.....	.....	.....
1942	5/19	40272	8/8	81	8/12	85	42996	8/14	87	8/20	93
1941	5/29	42336	8/14	77	8/14	77	.....	.....	.....	.....	.....
1940	5/30	38028	8/6	69	8/14	77	.....	.....	.....	.....	.....
1939	5/22	42804	8/17	79	8/15	77	.....	.....	.....	.....	.....
Aver. degree hours		42447	.....	.....	.....	.....	46951	.....	.....	.....	.....
		Iogreen 56					Ill. 14 x 13				
1949	5/27	49320	8/20	85	8/16	81	.....	.....	.....	.....	.....
1948	5/27	48384	8/28	93	8/27	92	.....	.....	.....	.....	.....
1947	6/4	52645	9/4	92	8/28	85	.....	.....	.....	.....	.....
1946	5/24	48036	9/1	100	9/1	100	46752	8/28	96	8/22	90
1945	5/24	47124	9/4	103	9/6	105	46368	9/2	101	8/30	98
1944	6/2	43116	8/20	79	9/1	91	.....	.....	.....	.....	.....
1943	5/28	46140	8/20	84	8/22	86	45816	8/19	83	8/15	79
1942	5/19	.....	.....	.....	.....	.....	43572	8/15	88	8/17	90
1941	5/29	.....	.....	.....	.....	.....	41952	8/13	76	8/18	81
Aver. degree hours		47832	.....	.....	.....	.....	44892	.....	.....	.....	.....

hybrids; Table 2, the white hybrids; and Table 3, the Country Gentlemen types.

The data from all three tables suggest a similar trend; the variation of the yearly degree hours of any hybrid from the average degree hours of the hybrid is too large for harvest date predictions or estimations. Likewise the actual number of days to harvest also varies significantly from year to year.

On examining the predicted date for Ioana, 8 of the 12 years were within 4 days of the predicted date, and only 4 out of 12 years were within 2 days of the predicted date. However, 4 days is too much since in hot weather the corn could pass from the prime stage of 72–73 per cent moisture to 65 per cent or less which would be standard or substandard corn. Predicted dates for Goldencross for 12 years are just as variable. Because of this too wide variation, degree days are not valuable in predicting harvest date.

Ioana, a golden hybrid, had for a 12-year average a degree hour

TABLE 3

DATE OF PLANTING, ACTUAL AND AVERAGE NUMBER OF DEGREE HOURS, AND THE ACTUAL AND PREDICTED DATE OF HARVEST AND DAYS TO HARVEST FOR COUNTRY GENLEMTEN HYBRIDS

Date of Planting		Degree Hours	Actual Hvst Date	Actual Days to Hvst	Predicted Hvst Date	Predicted Days to Hvst	Degree Hours	Actual Hvst Date	Actual Days to Hvst	Predicted Hvst Date	Predicted Days to Hvst
		Iogent 11					Iogent 27				
1947	6/4	53269	9/6	94	8/29	86	.....	.....	.....	.....	.....
1946	5/24	45000	8/23	91	9/4	103	47064	8/30	98	8/31	99
1945	5/24	45804	9/1	100	9/6	105	45804	9/1	100	9/4	103
1944	6/2	49464	9/5	95	9/3	93	49464	9/5	95	9/1	91
1943	5/28	50148	8/27	91	8/24	88	48108	8/24	88	8/23	87
1942	5/19	.....	.....	.....	.....	.....	45324	8/19	92	8/22	95
1941	5/29	.....	.....	.....	.....	.....	47604	8/25	88	8/25	88
1940	5/29	.....	.....	.....	.....	.....	48120	8/29	92	8/27	90
1939	5/30	.....	.....	.....	.....	.....	45816	8/24	86	8/28	90
1938	5/22	.....	.....	.....	.....	.....	51780	8/24	94	8/16	86
Aver. degree hours		48737	.....	.....	.....	.....	47676	.....	.....	.....	.....
		Illinois 8 x 6					Iogent 12				
1945	5/24	45804	9/1	100	8/31	99	.....	.....	.....	.....	.....
1944	6/2	47124	8/31	90	8/25	84	.....	.....	.....	.....	.....
1943	5/28	45924	8/20	84	8/19	83	.....	.....	.....	.....	.....
1942	5/19	42996	8/14	87	8/18	91	47220	8/21	94	8/21	94
1941	5/29	.....	.....	.....	.....	.....	42924	8/15	78	8/23	86
1940	5/29	.....	.....	.....	.....	.....	47304	8/27	90	8/26	89
1939	5/30	44376	8/20	82	8/22	84	46956	8/27	89	8/28	90
1938	5/22	47232	8/16	86	8/12	82	52416	8/25	95	8/15	85
Aver. degree hours		45576	.....	.....	.....	.....	47364	.....	.....	.....	.....

requirement of 42,939 degree hours. The extreme below this average was 38,100 degree hours or 4,839 degree hours less and the extreme above the average was 47,148 degree hours or 4,209 degree hours more. The spread in days to harvest was from 77 to 99.

The yearly estimates of days to harvest based on the average number of degree hours over a period of years for any variety is also unpredictable. For the Ioana variety this estimate varies from 12 days on the early side in 1945 to 12 days on the late side in 1950. Similar variations can be obtained for any of the hybrids listed in the tables.

Table 4 presents degree-hour data based on a date of planting experiment. Each year the planting period began about May 1 and continued at weekly intervals for 7 weeks or 42 days. The harvest period, based

TABLE 4

PLANTING DATES, HARVEST DATES, DAYS TO HARVEST, AND ACCUMULATED DEGREE HOURS FOR THREE SWEET CORN HYBRIDS IN 1937, 1938, AND 1939

1937				1938				1939			
Planting	Hvst	Days	Degree Hours	Planting	Hvst	Days	Degree Hours	Planting	Hvst	Days	Degree Hours
Goldencross Bantam											
5/1	8/2	94	41218	5/3	7/29	87	38555	5/2	7/22	81	39600
5/8	8/5	89	41986	5/10	8/2	84	40236	5/9	7/27	79	40404
5/15	8/9	86	43596	5/17	8/5	80	41736	5/16	8/2	78	42480
5/22	8/11	81	43380	5/24	8/5	73	39564	5/23	8/5	74	41100
5/29	8/14	77	42384	5/31	8/11	72	42736	5/30	8/12	74	40092
6/5	8/20	76	43308	6/7	8/17	71	42036	6/6	8/19	74	40056
6/12	8/27	76	45528	6/14	8/23	70	42840	6/13	8/25	73	39348
42–25 days				42–25				42–33			
191 x 1248 (Evergreen)											
5/1	8/6	98	44928	5/3	7/31	89	41064	5/2	7/26	85	38160
5/8	8/10	94	45358	5/10	8/5	87	42864	5/9	7/30	82	39456
5/15	8/17	92	48840	5/17	8/8	83	42960	5/16	8/4	80	40848
5/22	8/21	89	49896	5/24	8/13	81	43464	5/23	8/14	83	44328
5/29	8/24	87	48552	5/31	8/17	78	43224	5/30	8/20	82	45168
6/5	8/30	86	48996	6/7	8/23	77	43608	6/6	8/27	82	46032
6/12	9/6	86	41744	6/14	8/30	77	43944	6/13	9/3	82	46272
42–31 days				42–30				42–39			
Iogent 12											
5/1	8/12	104	48408	5/3	8/13	102	48504	5/2	8/3	93	46596
5/8	8/18	102	50734	5/10	8/17	99	50076	5/9	8/5	88	45384
5/15	8/23	98	52296	5/17	8/19	94	50328	5/16	8/14	90	48060
5/22	8/27	95	53112	5/24	8/20	88	48924	5/23	8/20	89	48372
5/29	8/30	93	52308	5/31	8/23	84	48312	5/30	8/27	89	46956
6/5	9/5	92	53352	6/7	8/30	84	49128	6/6	9/3	89	46620
6/12	9/10	90	53616	6/14	9/6	84	50340	6/13	9/8	87	47076
42–29 days				42–34				42–36			

on the 42-day period of planting for the three seasons, was 25 to 33 days for Goldencross, 30 to 39 days for 191 x 1248, and 29 to 36 days for Iogent 12. The reduction of the over-all harvest period ranged from 1½ to 2½ weeks of the over-all planting period. The number of days from planting to harvest decreased with the later dates of planting. Conversely, the degree hours required tended to increase with the later dates of plantings, with two exceptions — Goldencross and Iogent 12 in 1939.

Again with this data, it is obvious that factors other than temperature are involved in the development of sweet corn to canning stage of maturity.

DISCUSSION

From the data presented, it is suggested that the use of summated degree hours is of little practical value in predicting or estimating the harvest date of sweet corn. The variability ranges, on the upper and lower side of the average variety requirements, are too great for confidence. This is also true of the average number of days to maturity for any hybrid.

What then is the practical use of the degree-hours system? Evidently harvest date predictions are not possible because of seasonal variations. The other use of degree hours is to apply them at planting dates in the following manner. Average cumulative degree hours for a hybrid will be disregarded. However, the average number of degree hours per day during the general harvest season will be taken into account. Records of 20 years' duration at Ames indicate that there is only a 6°F. drop in the average daily temperature between August 12 and September 10. During this period of time perhaps 90 per cent of all the sweet corn planted in a specific year is harvested. The daily degree hours range from 552 on August 12 to 408 on September 10, with an average of 480 units per day.

The critical period of the planting season appears to be between May 15 and May 30. This critical period is indicated by the spread of approximately 7 days (Table 4) in the harvest season for these plantings. The ratio decrease in time is in this case 50 per cent compared to the over-all ratio time decrease of 20 to 35 per cent. It is in this period that the factory will be required to handle the most produce. Planning for this increased amount of material will greatly expedite movement in the field and in the plant.

Planting on a regular schedule of the above mentioned 480 degree hours per day will over the harvest period involve a rise in harvested acreage sometime during the middle of the estimated harvest period. This period where more corn matures than can be handled is the *glut* period that causes the difficulty in the plant. The system of using a longer daily degree-hour spread during the estimated glut period seems desirable and should tend to offset the 50 per cent time ratio increase during the critical time of harvest. This system would entail a graduated daily degree-hour spread to the approximate middle of the planting season,

then a decrease toward the end. Figures 1 and 2 show the theory in graphical representation.

The areas delineated by the planting dates on the harvest curve (Fig. 1) indicate the proportion of the planting that is harvested during August and September. Figure 2 shows the reverse relationship where the heat unit spread is varied to obtain, on the average, an evenly spread harvest period.

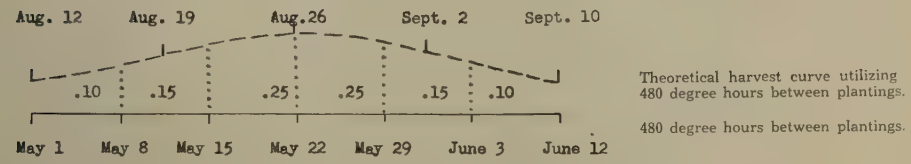

FIG. 1.—Theoretical harvest curve expected utilizing 480 degree hours between plantings.

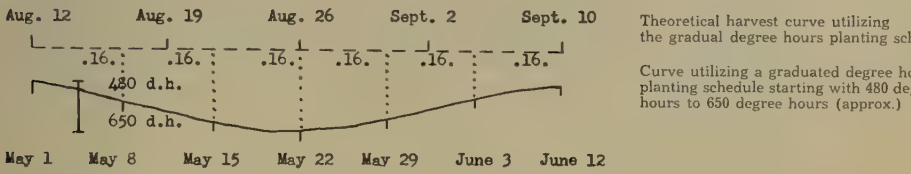

FIG. 2.—Theoretical harvest curve expected utilizing a graduated degree-hour planting schedule.

For example, if the first planting of sweet corn was on May 1, the second would not be made until approximately 480 degree hours had accumulated. The third planting would not be made until approximately 500 degree hours had accumulated. The fourth planting following the accumulation of 510 degree hours, and so on until the maximum degree-hour spread of 650 has been reached. The later plantings will then be made at decreasing degree-hour measurements until the end of the planting season.

Degree-hour measurement data can only be as accurate as the seasonal variation from the calculated normal. Before attempting use of the cumulative degree-hour system, the seasonal degree hours and the long time average for each variety must be determined. Comparison of the yearly requirement with the long time average will give the canner factual information on the extremes for variation. It is entirely possible that the degree-hour summation system will prove more efficient

at certain latitudes, and canners in these latitudes can use them with a greater degree of accuracy.

Complete dependence upon degree hours will not serve the prediction purpose entirely. The total number of degree hours are dependent upon too many other natural factors (slope, soil type, light, moisture, etc.). Degree hours can be used only as a rough guide. The work of the field man in checking his fields cannot be reduced, and periodic investigations over the growing period still remain a necessity, particularly after silking time.

SUMMARY AND CONCLUSIONS

1. The average number of degree hours and the actual degree hours were accumulated for eleven sweet corn hybrids. Degree-hour accumulations based on planting dates within a season were also obtained.

2. The variation of degree hours between and within seasons suggested that the average degree-hour requirements of each variety could not be used to predict date of harvest from the date of planting.

3. The critical period of planting appears to be within May 15 and 30. All of the corn planted within this two-week period matures for harvest within a period of approximately one week.

4. The most efficient use of the degree-hour summation theory lies in scheduling plantings and not in predicting harvest dates. A method is suggested for using a gradated method of degree-hour accumulation for determining planting schedules.

5. Dependence upon degree hours will serve as a rough guide for planting schedule purposes.

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PRODUCTION OF GLYCEROL BY FERMENTATION

I. Fermentation of Dextrose

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Received July 26, 1951

Glycerol, also called glycerin or propanetriol, is a viscous, odorless and colorless liquid having a slightly sweet taste. It is a trihydric alcohol with the chemical formula: $CH_2OH \cdot CHOH \cdot CH_2OH$. As a chemical raw material it has many uses in such diversified products as explosives, textiles, foods, beverages, cosmetics, plastics, paints and protective coatings, to mention but a few. Leffingwell and Lesser (13) have included in their excellent book on the industrial and commercial applications of glycerol a list of 1,583 individual uses for glycerol, and this list is doubtless incomplete.

The principal commercial source of glycerol has always been from saponification of fats and oils in making soaps. As a by-product commodity, glycerol has frequently been in short supply, especially during war periods when household fat salvage projects have emphasized the need. Even when war emergencies have not existed, glycerol shortages have sometimes appeared, and the wide variety of applications would lead to expanded consumption of glycerol if it were available in unlimited quantities at reasonable cost.

Glycerol may also be produced on the industrial scale by synthesis from hydrocarbon gases or by the fermentation of carbohydrates. A new synthetic plant which came into production in 1948 has assured a more adequate industrial supply of pure glycerol (11). Commercial production by fermentation has been limited to operations in Germany during World War I when the requirement of glycerol for explosives manufacture overcame considerations of cost. In Germany during that period, 24 factories were operated, producing over 1,000 tons of dynamite glycerol per month. The process employed in Germany involved yeast fermentation of pure beet sugar in media containing large quantities of sodium sulfite. The fermentations were satisfactory, but the recovery of the glycerol from the fermented beers was exceedingly difficult and very inefficient. May and Herrick (15) briefly reviewed the German experience and stated that about 10 to 12 kg. of sugar were required to produce 1 kg. of dynamite glycerol.

Due to occasional shortages and high prices of glycerol, considerable interest in fermentative production of glycerol developed after World

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War I. An indication of the interest is the listing of 114 patents on the subject by Whalley (20) in her list of abstracts of articles and patents, through 1941, on the production of glycerol by fermentation. The processes described in these patents involve modification of the normal yeast fermentation by addition of either soluble sulfites or alkaline reagents.

The problem of recovering the glycerol from fermented beers has never been fully solved, due principally to the fact that in the processes proposed large amounts of soluble salts have been required to obtain increased glycerol production by yeast fermentation. Usually the fermented beers contain several times as much soluble impurities as glycerol. The recovery problems would largely be obviated if the soluble impurities in fermentation beers could be greatly reduced so that conventional procedures, distillation methods such as are used in recovery from spent soap lyes, or perhaps solvent extraction procedures, might be used for obtaining the glycerol. In an effort to evolve a workable process which would materially decrease the content of soluble salts, considerable research has been devoted in our laboratories over a period of several years to the problem of glycerol fermentation. The results are available only as typewritten doctoral theses (5, 9, 12), and the fundamental information obtained seems worthy of summarizing in the present series of two papers.

THEORY OF GLYCEROL FERMENTATION OF SUGARS

Glycerol was first identified as a product of yeast fermentation by Pasteur (17) while studying the alcoholic fermentation of wines and beers. He found that in all normal alcoholic fermentations about 3 g. of glycerol were produced for each 100 g. of sugar fermented by yeast.

Shortly before World War I much interest arose in the mechanism of alcoholic fermentation. Neuberg and his co-workers made important contributions in this field, and in 1920 Neuberg, Hirsch and Reinfurth (16) proposed three forms of sugar dissimilation by yeast:

I. $C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$ (normal alcohol fermentation);

II. $C_6H_{12}O_6 \longrightarrow CH_3CHO + CO_2 + C_3H_8O_3$ (sulfite fermentation);

III. $2\ C_6H_{12}O_6 + H_2O \longrightarrow 2\ CO_2 + CH_3COOH + C_2H_5OH + 2\ C_3H_8O_3$ (alkaline fermentation).

Neuberg's first form of fermentation, the normal alcohol fermentation, is generally known as the Gay-Lussac reaction. His second form was demonstrated by the addition of sodium sulfite to an active yeast fermentation. In this manner acetaldehyde was fixed by the sulfite, and for each mol of acetaldehyde bound, a mol of glycerol was found. The third form may be considered as merely a modification of the second form wherein the alkali added results in causing acetaldehyde to undergo the Canizzaro reaction to form acetic acid and ethanol.

In more recent years a great many workers have contributed to the elucidation of the mechanism of yeast fermentation. Hence, our present knowledge of the complex series of chemical reactions and the enzymes involved in yeast fermentation is more complete than for any other

fermentation process, and is usually designated the "Embden-Meyerhof-Parnas scheme for yeast dissimilation." According to this scheme, glucose is first phosphorylated in three steps yielding fructose-1,6-diphosphate, which breaks down into two triose phosphates, dihydroxyacetone phosphate and 3-glyceraldehyde phosphate, which are in equilibrium. Under ordinary conditions the glyceraldehyde phosphate is further transformed through several steps into pyruvic acid, and this then into acetaldehyde which is hydrogenated to ethanol. In the case of sulfite fermentation, the acetaldehyde is bound in the form of the bisulfite addition product which is not reducible, and then the hydrogen which would otherwise reduce acetaldehyde reduces glyceraldehyde phosphate to α-glycerophosphate which in turn is dephosphorylated to glycerol. In the initial phases of normal alcoholic fermentation, before much acetaldehyde is formed to act as primary hydrogen acceptor, the glyceraldehyde phosphate is reduced to glycerol, which accounts for the approximate 3 per cent of glycerol normally found in alcoholic fermentations.

During World War I both Neuberg's second and third forms of fermentation were made the bases for fermentation processes for the production of glycerol. Connstein and Lüdecke (1) investigated both processes, and developed the procedure used in Germany on the industrial scale. This process employed yeast fermentation of pure beet sugar mashes containing 10 per cent of sugar and 4 per cent of sodium sulfite, with small amounts of inorganic salts added as yeast nutrients. In the United States a glycerol fermentation process was also developed, described by Eoff, Lindner and Beyer (4), and tested on the semi-commercial scale. In this process 17.5 to 20 per cent sugar solutions, obtained by diluting blackstrap molasses, were fermented by adapted strains of wine yeast in the presence of 5 to 6 per cent of sodium carbonate.

Shortly after the war, patents were issued to Connstein and Lüdecke (2) and to Eoff (3) on these processes, and subsequently many other patents on glycerol fermentation processes have been granted (20). The processes of these many patents involve variations in method, using alkaline salts such as sodium carbonate and others, and soluble sulfites such as sodium sulfite and sodium sulfite-bisulfite mixtures. All of these fermentation processes involve fermentation under alkaline conditions in the presence of large quantities of soluble materials. These conditions are not favorable for greatest yeast activity nor for recovery of the glycerol produced.

MATERIALS AND METHODS

In order to study the production of glycerol in a simple medium of known composition, a semi-synthetic medium involving the minimum of dissolved ingredients other than sugar was developed. This medium gave maximum rapid dissimilation of dextrose by normal yeast fermentation and was used as the basal medium for all the experiments reported herein unless otherwise stated. The composition of this basal medium

was: Dextrose 5 to 15 g., yeast extract 0.375 g., NH_4Cl or urea 0.15 or 0.084 g., $K_2HPO_4 \cdot 3H_2O$ 0.075 g., KH_2PO_4 0.075 g., $MgSO_4 \cdot 7H_2O$ 0.02 g., $CaCl_2$ 0.01 g., tap water to make 100 ml. final volume.

The dextrose was obtained from the Pfanstiehl Chemical Co. In most cases the technical anhydrous grade was used, but in a few cases the C. P. anhydrous grade was employed. The yeast extract was the Difco powder. All other ingredients were of the ordinary laboratory reagent grade.

Since the media varied in sugar content, the dextrose concentration for each experiment will be specified. Unless designated otherwise the media contained ammonium chloride rather than urea. For some of the later experiments instead of yeast extract concentrated corn steep liquor, obtained from American Maize-Products Co., was used in the concentration of 0.4 ml. per 100 ml. of medium. In these cases the salts were also usually omitted. These variations will be noted as the individual experiments are described.

The culture of yeast used was a strain of distillery yeast, listed in the collection of this laboratory as No. 43. The yeast was cultivated for use as inoculum for experimental fermentations in the semi-synthetic medium containing 10 per cent dextrose, and incubated at 30°C. Unless otherwise indicated an active 24-hour culture of this yeast was used for inoculating the experimental fermentations, employing an inoculation ratio of 10 per cent. For some of the fermentations, as will be indicated, commercial Fleischmann yeast cakes were used for inoculation. All fermentations, unless otherwise stated, were incubated at 30°C.

Methods for the direct determination of glycerol are time-consuming, and in most of these procedures other materials which would be present in a fermented medium interfere with the analysis. Hence, a number of procedures were tested and a method was developed involving oxidation with ceric sulfate. Details of this method have been published by Fulmer, Hickey and Underkofler (6) and this procedure was used for analysis of glycerol for the work reported in this paper unless otherwise stated. However, it was found during the course of work involving actual isolation of glycerol from fermented beers that the maximum recoveries were always somewhat lower than the yields calculated from the ceric sulfate oxidations. Further investigation showed that an iodine titration method, which was an adaptation of the procedure described by Tomoda (18), gave glycerol analyses which were comparable with glycerol yields obtained by actual recoveries. This method does not determine glycerol directly, but rather measures the amount of sulfite fixed by acetaldehyde and, hence, is applicable only to sulfite fermentations. Since one mol of glycerol is produced in the fermentation for each mol of acetaldehyde bound, the determination of fixed sulfite is a measure of the glycerol produced. Undoubtedly the results, calculated as glycerol, are slightly low, but its rapidity and the close correspondence of the results of this method with actual glycerol recoveries make it most useful. The procedure is much simpler and more rapid than the ceric

sulfate method, and was generally employed for the later work. The details of the method are as follows:

Into a 125-ml. Erlenmeyer flask are measured 5 ml. of fermentation beer to be analyzed to which 0.5 ml. of 6 *N* hydrochloric acid and 0.5 ml. of 1 per cent soluble starch solution are added. The contents of the flask are titrated with 0.1 *N* iodine solution to the appearance of the first purple color that persists for 15 seconds. The amount of iodine consumed is a measure of the free sulfite present. Solid sodium bicarbonate is then added from a spatula, a little at a time, until effervescence ceases and solid sodium bicarbonate remains undissolved. The mixture is finally titrated with standard 0.1 *N* iodine solution to the first appearance of persistent slight purple tinge, the exact volume of iodine solution required being noted. This volume is equivalent to the amount of sulfite bound by the acetaldehyde, which in turn is equivalent to glycerol formed. Since the equivalent weight of sulfite is one-half the molecular weight, the equivalent weight of glycerol is one-half the molecular weight, and the weight of glycerol for the 5 ml. sample titrated is therefore:

$$\text{g. glycerol} = \text{ml. } 0.1\ N \text{ iodine} \times 0.0046.$$

Where sugar analyses were made the method of Underkofler, Guymon, Rayman and Fulmer (19) was employed.

A number of the experimental fermentations were run at controlled *p*H levels. This was accomplished by the use of a Cameron *p*H Recorder-Controller. This instrument employed a glass electrode system and continually recorded the *p*H of the medium on a moving chart. It was so designed that it could control the automatic addition of reagent as required to maintain the *p*H at the desired level.

EXPERIMENTAL

Since previously described glycerol fermentation processes all involve yeast fermentation under alkaline conditions in the presence of large amounts of soluble salts which interfere with glycerol recovery, attention was turned to the possible use of reagents which could easily be removed after completion of the fermentation. Substances which theoretically might effectively increase glycerol yields and could be removed readily are ammonium sulfite, ammonium hydroxide, and slightly soluble sulfites such as calcium sulfite and magnesium sulfite.

Alkaline Fermentations

Sodium carbonate was recommended by Eoff (3) as an agent to bring about the formation of increased amounts of glycerol from the altered alcoholic fermentation. Eoff, Lindner and Beyer (4) and also McDermott (14) stated that addition of solid sodium carbonate rather than solutions was necessary to secure maximum yields of glycerol.

A test was made on the effectiveness of sodium carbonate in producing glycerol when added automatically as a saturated solution (150 g.

anhydrous salt per liter) by means of the Cameron Recorder-Controller at a controlled *p*H of 7.5. The semi-synthetic medium contained urea and 15 per cent dextrose. Three liters of medium were prepared, sterilized and inoculated with 300 ml. of active yeast culture. Incubation was at 32°C. The *p*H was controlled at 6.8 for 20 hours to allow good growth of yeast, then changed to a *p*H of 7.5 over a period of 2 hours, and maintained at that value by automatic addition of the sodium carbonate solution as required. Fermentation was complete in about 90 hours, and analysis was made at 96 hours. Addition of 1,977 ml. of the sodium carbonate solution, corresponding to 296 g. of sodium carbonate, to the original 3,300 ml. of inoculated fermentation medium was required to maintain the *p*H at 7.5. This diluted the original sugar content of 15 per cent to about 9.4 per cent based upon final volume. Fermentation of the sugar was 99.6 per cent complete, and the glycerol yield was 10.2 per cent of the sugar weight. This rather low yield was undoubtedly due to excessive dilution of the medium, since Eoff, Lindner and Beyer (4) and McDermott (14) found glycerol yields were poorer in more dilute solutions.

Another fermentation was run in a similar manner but substituting a solution containing 50 g. of sodium hydroxide per 100 ml. for control of the *p*H. The semi-synthetic medium contained 15 per cent dextrose. The original volume was 1,500 ml. and 200 ml. of 48-hour yeast culture was used for inoculation. Over a period of 6 hours after inoculation the *p*H was gradually increased to 8.0, and then controlled at that level. After 118 hours the fermented beer was analyzed. The final volume was 1,875 ml., and 175 ml. of the sodium hydroxide solution, corresponding to 87.5 g. of sodium hydroxide, had been used in maintaining the *p*H. Sugar utilization was 97.5 per cent, and the glycerol yield was 22.8 per cent of sugar weight.

The fermentations at controlled *p*H with sodium carbonate and sodium hydroxide verified the findings of previous workers that fermentation under alkaline conditions results in increased glycerol production by yeast fermentation. Attempts were next made to conduct fermentations at controlled alkaline *p*H levels maintained by addition of ammonium hydroxide, since ammonium compounds could presumably be easily removed by volatilization after fermentation as contrasted with the other soluble alkalis. A fermentation was set up with the *p*H controlled at 7.5 by addition of ammonium hydroxide solution. Fermentation ceased almost immediately under these conditions.

A second fermentation using semi-synthetic medium containing 15 per cent dextrose was run in which the *p*H was controlled at 6.8 by automatic addition of ammonium hydroxide solution. After 96 hours 99.5 per cent of the dextrose had been fermented and the glycerol yield was 13.8 per cent of the sugar weight.

Two other attempts were made to conduct fermentations controlled at *p*H 7.7 and *p*H 7.5 by addition of ammonium hydroxide. In both cases fermentation was quickly inhibited. Other small-scale laboratory fer-

mentations also failed when the alkalinity, in the presence of ammonium compounds, was greater than a *p*H of 7.0. This difficulty was undoubtedly associated with the presence of ammonium hydroxide, rather than the alkaline *p*H itself, since fermentations made alkaline with sodium carbonate or sodium hydroxide went to completion.

An extensive investigation of the effect of the concentration of ammonium salts and of *p*H was therefore conducted. Different ammonium salts, including the chloride, nitrate, sulfate and carbonate were employed at varying concentrations and at different *p*H levels. Similar results were obtained with each of these ammonium salts. In all cases where the *p*H was 7.0 or higher fermentations were inhibited. Representative data for one fermentation series are given in Table 1. The fermentations for this series were conducted in 500 ml. Erlenmeyer flasks, each containing 300 ml. of semi-synthetic medium of 15 per cent dextrose content to which had been added the indicated quantities of ammonium carbonate, and the *p*H adjusted to the approximate initial values given by addition of required amounts of hydrochloric acid. Each fermentation flask was inoculated with 22 ml. of active yeast culture, and the fermentations were incubated for 6 days.

The data show that no fermentation occurred where the initial *p*H was above 7, and that practically complete utilization of the sugar occurred in all fermentations where the initial *p*H was below 7. Glycerol

TABLE 1

FERMENTATION OF DEXTROSE IN MEDIA OF VARYING *p*H AND VARYING LEVELS OF AMMONIUM CARBONATE

$(NH_4)_2CO_3$ Added, g. per 100 ml.	NH_4^+ Normality	Initial *p*H	Final *p*H	Yields, Per Cent on Sugar Weight		Residual Sugar, Per Cent
				Ethanol	Glycerol	
0.0000	0.0282	5	2.86	42.9	4.14	0.26
0.0898	0.0469	6	3.59	42.8	4.51	0.26
0.224	0.0750	6	3.94	41.6	5.53	0.23
0.404	0.1124	6	4.32	40.2	5.80	0.23
0.580	0.149	6	4.40	39.8	7.23	0.23
0.763	0.179	6	5.00	39.5	7.48	0.24
1.210	0.280	6	6.37	39.8	8.50	0.26
1.66	0.374	8	7+	no fermentation		high
2.56	0.561	8	7+	no fermentation		high
3.46	0.748	8	7+	no fermentation		high
4.36	0.935	8	7+	no fermentation		high

yields increased with increasing ammonium content in the *p*H range where fermentations occurred.

To investigate whether or not the inhibiting effect, observed in all fermentations attempted in the alkaline range when ammonium salts were employed, was one of ammonium concentration, an experiment was conducted to investigate sugar utilization in fermentations containing various concentrations of ammonium sulfate with the *p*H adjusted to different levels by means of sodium carbonate. The semi-synthetic medium containing urea and 15 per cent dextrose was employed, using 200 ml. in each 300 ml. Erlenmeyer flask, with requisite amounts of ammonium sulfate added. After sterilization all media were inoculated with 20 ml. of active yeast culture, and were incubated for 100 hours. The *p*H of each medium was adjusted by frequent additions of solid sodium carbonate as required. The results obtained are shown in Figure 1.

It is obvious from the figure that at constant *p*H near or above 7, increasing the ammonium concentration decreased utilization of the dextrose. It may also be concluded that at constant ammonium concentration, increasing *p*H decreased sugar dissimilation. However, at the lowest *p*H value, 6.5, dissimilation was essentially complete at all levels of ammonium concentration. The inhibitions found at higher *p*H levels lead to the conclusion that this toxicity is a function of the concentration of ammonia or of ammonium hydroxide, since these will be formed in accordance with the equilibria,

$$NH_4^+ + OH^- \rightleftharpoons NH_4OH \rightleftharpoons NH_3 + H_2O,$$

and neither the ammonium ions (in acid solutions) nor hydroxide ions (in alkaline fermentations not containing ammonium) are toxic in themselves.

The results of the experiments with ammonium hydroxide and ammonium salts proved that the use of ammonium hydroxide or other ammonium agents for alkaline fermentations was impractical.

Sulfite Fermentations

For the production of glycerol by yeast fermentation, in accordance with the mechanism of the fermentation, intermediate acetaldehyde as it is formed must be rendered incapable of reduction. This is most easily accomplished by fixing the acetaldehyde by means of bisulfite. However, the bisulfite ion concentration must be kept below that which is detrimental to yeast activity, or the fermentation will completely cease. With soluble sulfites alkaline conditions are necessary in order not to exceed the inhibiting level of bisulfite.

Soluble sulfites, such as sodium sulfite, under alkaline conditions effectively increase glycerol yields, but enhance the difficulty of recovering the glycerol after completion of the fermentation because they cannot readily be removed. If removed by precipitation they are simply replaced in the residual liquor by other soluble salts. Sulfites which could be readily removed without leaving soluble residues are restricted to am-

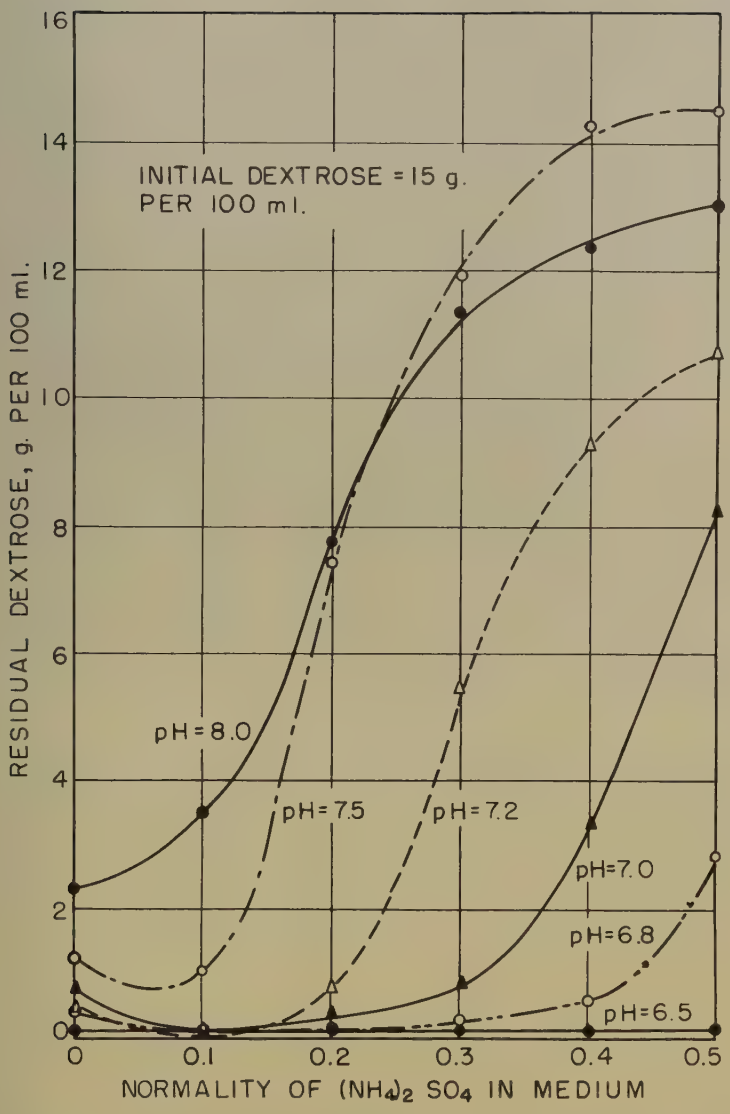

FIG. 1.—Sugar consumption in fermentations as related to *p*H and ammonium concentration.

monium sulfite and insoluble sulfites such as those of calcium or magnesium. Since ammonium sulfite is very soluble, to decrease the concentration of bisulfite below the inhibiting level would probably require alkaline conditions, and as noted above yeast fermentations under alkaline conditions in the presence of ammonium compounds have not been found possible. However, a few trials in using ammonium sulfite were made. Fermentations were attempted in semi-synthetic media containing 15 per cent dextrose in 300 ml. quantities, to which 10 g. of ammonium sulfite were added. The *p*H of the medium after addition of the sulfite was found to be 7.4. Flasks of the medium containing ammonium sulfite were adjusted to *p*H levels of 7.0 and 6.5. Two flasks of media at each of the *p*H levels 7.4, 7.0 and 6.5 were inoculated with active yeast culture, using 30 ml. for each. No fermentation occurred in any of the flasks. Evidently under alkaline conditions the ammonium hydroxide toxicity prevented fermentation while at the neutral and acid reactions the bisulfite tolerance of the yeast was exceeded. In other experiments small amounts of ammonium bisulfite were added during the progress of fermentation. The maximum amount of ammonium sulfite which could be added in this manner without almost completely inhibiting the fermentations was about 3 to 4 g. per 100 ml. Yields of glycerol over the controls to which no sulfite had been added were increased somewhat, and in one instance the yield reached 15.7 per cent on sugar weight. However, all fermentations were slow and very erratic, and the method did not appear promising.

Attention was therefore turned to the possible use of the slightly soluble sulfites of calcium and magnesium. With these sulfites, the bisulfite concentration may be controlled by varying the *p*H of the solution in accordance with the equilibrium

$$SO_3^{=} + H^+ \rightleftharpoons HSO_3^-.$$

Experiments were therefore undertaken to ascertain the glycerol yields which could be obtained using calcium sulfite or magnesium sulfite in excess of their solubility limits, with the *p*H controlled at various levels.

Solubility tests were made with calcium sulfite and magnesium sulfite at different *p*H levels, both with and without addition of acetaldehyde. From these tests the useful range of calcium sulfite for acetaldehyde fixation seemed to be *p*H of about 5.0 to 6.0 and for magnesium sulfite *p*H of about 6.0 to 7.0.

Preliminary experiments were conducted using the semi-synthetic medium containing 15 per cent dextrose. The medium was distributed in 200 ml. quantities in 300 ml. Erlenmeyer flasks and sterilized. Each flask was inoculated with 20 ml. of active yeast culture and solid calcium sulfite added, equivalent to 20 g. of anhydrous calcium sulfite per flask. Anhydrous calcium sulfite was added to half of the flasks, and hydrated calcium sulfite to the other half. The *p*H was adjusted to the determined levels in the various flasks by addition of sulfuric acid and all flasks were incubated for 96 hours. The flasks were shaken occasionally by hand,

and the *p*H adjusted to the original levels at intervals by addition of sulfuric acid. The results are shown in Table 2.

From the results of this experiment it seemed that the limiting *p*H was about 5.0, and hydrated calcium sulfite gave better results than the anhydrous salts. None of the yields obtained could be considered satisfactory, but it was thought that the yield might have been improved by use of higher levels of sulfite and by continuous agitation. An apparatus

TABLE 2

GLYCEROL PRODUCTION FROM DEXTROSE IN THE PRESENCE OF CALCIUM SULFITE AT VARYING *p*H

*p*H	Calcium Sulfite Used	Residual Dextrose, Per Cent	Glycerol Dextrose Utilized	Yield, Per Cent on Total Dextrose
4.0	Anhydrous	80.6	24.7	4.79
4.0	Hydrated	84.0	27.8	4.45
4.5	Anhydrous	3.06	11.00	10.66
4.5	Hydrated	86.0	26.4	3.70
5.0	Anhydrous	0.94	10.31	10.20
5.0	Hydrated	0.89	14.23	14.01
5.5	Anhydrous	0.54	10.68	10.68
5.5	Hydrated	0.86	12.38	12.27
6.0	Anhydrous	0.54	10.39	10.31
6.0	Hydrated	0.59	11.39	11.31

was therefore designed for stirring media in six flasks continuously and simultaneously.

A series of fermentations were run with *p*H adjusted to 4.7, 4.8, 4.9, 5.0 and 5.5. Each flask contained 200 ml. of semi-synthetic medium of 15 per cent dextrose content and moist hydrated calcium sulfite equivalent to 60 g. of the anhydrous salt. The *p*H of the medium in each flask was adjusted to the determined value by addition of sulfuric acid, and each flask was then inoculated with 20 ml. of active yeast culture. The fermentations were stirred continuously at 26°C. for 4 days. Fermentation occurred only in the flasks adjusted to *p*H 5.0 and 5.5. The glycerol yields were 15.1 and 12.2 per cent on sugar weight, respectively, in these fermentations.

A second series was set up in the same way, but each fermentation medium was inoculated with one-fifth of the cake of calcium sulfite and yeast obtained from the previous two successful fermentations. Additional hydrated calcium sulfite was added so that the total amount in each flask was equivalent to 60 g. of the anhydrous salt. The media were adjusted to the determined *p*H levels by addition of sulfuric acid. No fermentation occurred at *p*H 4.5, but excellent fermentation took place in

the media at *p*H 4.7, 4.9, 5.1 and 5.3. Residual sugar ranged from 0.42 to 0.52 per cent and the glycerol yields were practically constant at 10.01 to 10.06 per cent of the dextrose weight.

Following these investigations on fermentations in the presence of calcium sulfite, yeast fermentations were run in the presence of magnesium sulfite ($MgSO_3 \cdot 6H_2O$) with special attention to *p*H, glycerol yields, and initial sugar concentrations. Since magnesium sulfite is somewhat more soluble than calcium sulfite, higher acetaldehyde fixation might be expected than with calcium sulfite. Two preliminary fermentations were run, using in each case 200 ml. of semi-synthetic medium containing 10 per cent dextrose, to which were added 50 g. of hydrated magnesium sulfite. The flasks were inoculated with the yeast-calcium sulfite cake from the previous fermentation. The initial unadjusted *p*H of the media after addition of the magnesium sulfite was about 7.0. After fermentation for 24 hours the *p*H had dropped to 6.2–6.3. At about 50 hours the *p*H had increased to 7.1, and the media were quiescent. Analyses were made four days after inoculation. The yields of glycerol were 22.05 and 22.2 per cent on dextrose weight, respectively. These preliminary experiments indicated that magnesium sulfite is more efficient than calcium sulfite in influencing the production of glycerol.

To study the effect of *p*H on the fermentation in the presence of magnesium sulfite, six flasks each containing 200 ml. of semi-synthetic medium containing 10 per cent dextrose were prepared. To each was added 60 g. of magnesium sulfite hydrate, and each was inoculated with a portion of yeast-magnesium sulfite cake from the previous fermentation. The *p*H of each medium was adjusted to the desired level by addition of a few drops of 7.5 *N* sulfuric acid as required. The media were stirred continuously at 26°C. The *p*H level in the various flasks was maintained by frequent addition of sulfuric acid as required. The fermentations were all vigorous and were analyzed after 4 days. The results are given in Table 3. The data indicate that good fermentations occurred throughout the range of *p*H studied, and the glycerol yields were practically constant.

In the above described experiments with fermentations in the presence of calcium sulfite or magnesium sulfite, semi-synthetic media containing 10 or 15 per cent of dextrose were employed. It was of interest to determine whether there is a relationship between initial sugar concentration and glycerol yield in these fermentations. Several series were run with somewhat contradictory results, but in general conversion of sugar to glycerol increased with decreasing sugar concentration. In a typical series semi-synthetic medium containing varying dextrose contents was used, employing 200 ml. of medium to which were added 60 g. of solid calcium sulfite hydrate per flask. All media were adjusted to an initial *p*H of 5.5. The media were inoculated with yeast-calcium sulfite cake from a previous fermentation, and were continuously stirred at 26°C. for 4 days before being analyzed. In a second series the procedure was the same except that 40 g. of magnesium sulfite hydrate per

flask were added and each flask of medium inoculated with 20 ml. of yeast culture grown in semi-synthetic medium to which a little magnesium sulfite had been added. The initial *p*H of this series was about 7.0. The results of the analyses of these series are given in Table 4.

The data indicate that the fermentations were essentially complete after the four day period, and in each series the percentage conversion of sugar to glycerol increased with decreasing sugar concentration. This effect was considerably greater in the case of the calcium sulfite fermentation.

Another fermentation series was run in duplicate in which semi-synthetic media of varying dextrose contents were used, employing 300

TABLE 3
GLYCEROL PRODUCTION FROM DEXTROSE IN THE PRESENCE OF MAGNESIUM SULFITE AT VARYING *p*H

*p*H	Residual Dextrose, g. per 100 ml.	Glycerol Yield, Per Cent on Dextrose
5.5	0.14	23.3
5.8	0.14	23.1
6.0	0.12	22.4
6.3	0.13	22.5
6.5	0.13	22.7
7.0	0.13	23.8

ml. of medium in each flask to which was added an amount of magnesium sulfite hydrate equal to the weight of the sugar present. The initial *p*H of the media was about 7.0, and each was inoculated with 30 ml. of active yeast culture. The contents of the flasks were shaken occasionally and samples were taken from each for analysis after 5, 7 and 21 days. The glycerol analyses for this series were made by the iodine titration method. The results, as averages of the duplicate fermentations, are given in Table 5. It is apparent from the data that for short periods of fermentation the glycerol yields were superior with lower sugar concentrations as found in the previous series. However, upon prolonged incubation the yields of glycerol were essentially the same from all levels of dextrose concentration. The fermentations with sugar concentration above 12 per cent were much slower, and too prolonged to be practical. It was concluded from the experiments attempted that a dextrose concentration of about 10 per cent was most satisfactory from the standpoint of highest yields in a reasonable fermentation period.

The experiments on the fermentation of dextrose in the presence of calcium sulfite and magnesium sulfite showed much better glycerol pro-

TABLE 4
GLYCEROL PRODUCTION IN MEDIA CONTAINING VARYING DEXTROSE CONCENTRATIONS

Sulfite Used	Initial Dextrose, g. per 100 ml.	Residual Dextrose, g. per 100 ml.	Glycerol Yield, Per Cent of Dextrose
$CaSO_3$	4	0.084	20.8
$CaSO_3$	8	0.084	16.5
$CaSO_3$	12	0.056	12.6
$CaSO_3$	16	0.096	9.8
$MgSO_3$	4.5	0.127	23.8
$MgSO_3$	9.0	0.080	22.4
$MgSO_3$	13.5	0.080	20.4
$MgSO_3$	18.0	0.121	18.5
$MgSO_3$	22.5	0.126	17.6

duction with the latter. As reported above, the successful fermentations in the presence of calcium sulfite regularly gave glycerol yields of the order of 10 per cent, whereas successful magnesium sulfite fermentations gave glycerol yields of the order of 20 per cent on dextrose weight. Variation of conditions did not significantly change the yields of glycerol obtained. Since magnesium sulfite is somewhat more soluble than calcium sulfite it seemed possible that the glycerol yields were dependent upon the total sulfite in solution. Previous work (8) with varying concentrations of sodium sulfite has shown this to be true for this salt.

Analysis of magnesium sulfite fermentations showed the total concentration of the sulfite present in solution to be about 2.6 g. per 100 ml. This amount of magnesium sulfite is equivalent to about 3.1 g. of sodium sulfite. With sugar concentrations of 10 per cent, this amount of sulfite would be in the neighborhood of 30 per cent sulfite on sugar by weight.

TABLE 5
GLYCEROL PRODUCTION IN MEDIA OF VARYING DEXTROSE CONCENTRATIONS AFTER DIFFERENT PERIODS OF INCUBATION

Initial Dextrose, g. per 100 ml.	Glycerol Yield, Per Cent on Dextrose		
	5 days	7 days	21 days
9	17.1	19.3	18.3
12	14.3	17.8	18.1
15	11.1	15.0	19.5
16	8.6	12.1	18.6

Previous investigators have emphasized the increased glycerol yields from sucrose obtained by addition of increasing amounts of sodium sulfite. For example, Gehle (8) gave the following results:

Per cent sodium sulfite on sugar	Per cent glycerol formed from sugar
25	17.9
33	21–23
50	24–26
75	29–32
100	33
133	35

The amount of glycerol obtained from the magnesium sulfite fermentations, containing the equivalent of about 30 per cent sodium sulfite, was in the neighborhood of 20 per cent, which agrees remarkably well with the yield given by Gehle at about this sulfite level. It seemed, therefore, of interest to investigate further the fermentation of dextrose in the presence of sodium sulfite.

Using the semi-synthetic medium containing 10 per cent dextrose, in 300 ml. portions in 500 ml. Erlenmeyer flasks, to each of two flasks were added 15, 30 and 45 g. of sodium sulfite, respectively. These amounts corresponded to 50, 100 and 150 per cent on the weight of sugar. Each flask was inoculated with 30 ml. of active yeast culture and incubated at 30°C. Fermentation was feeble in the flask containing the lowest amount of sulfite, and there was no apparent fermentation in the other flasks. Glycerol yields by the iodine titration method averaged 17.9, 2.6 and 0.3 per cent of the sugar weight after 5 days incubation. Microscopic examination showed that little or no yeast proliferation had occurred in these fermentations. Since in most of the previous work on sodium sulfite fermentations massive inoculations had been employed (German commercial operations during World War I used press yeast), a study was made on the size of inocula as they affect glycerol production from dextrose medium containing sodium sulfite. Preliminary experiments showed that with large yeast inocula, addition of nutrients such as employed in the semi-synthetic medium did not improve glycerol yields. Hence, for most of the work the sugar solutions were made up with tap water and the only nutrient supplied was corn steep water.

When ordinary inocula of 10 per cent by volume of active yeast culture in semi-synthetic medium were employed, counts showed that the maximum number of yeast cells present in the inoculated media was about 7 million cells per ml., and the usual level was about 5 million cells per ml. Flasks of media were prepared, each 300 ml. Erlenmeyer flask containing 200 ml. of solution made with tap water and containing 10 g. dextrose, 10 g. sodium sulfite and 0.4 ml. of steep liquor concentrate per 100 ml. Duplicate flasks were inoculated with measured portions of a suspension of Fleischmann yeast cakes in distilled water. After fermentation for 4 days at 30°C. the media were analyzed by iodine titration

for glycerol yields. Results are given in Table 6. The data showed that use of large inocula resulted in increased glycerol production from 10 per cent dextrose solutions containing 10 per cent sodium sulfite. Glycerol yields did not increase appreciably when the initial yeast count was over about 100 million cells per ml. On the other hand the poor yield from the fermentation initially inoculated with 9 million cells per ml. showed that the usual inoculations employing 5 to 7 million cells per ml. would definitely not be sufficient to bring about satisfactory fermentations of dextrose in solutions of high sulfite concentrations.

TABLE 6

EFFECT OF INITIAL YEAST COUNT UPON GLYCEROL PRODUCTION FROM MEDIA CONTAINING DEXTROSE AND SODIUM SULFITE

Fraction of Yeast Cake	Approximate Initial Yeast Count, Millions per ml.	Glycerol Yields, Per Cent on Dextrose
0.01	9	5.3
0.05	46	28.8
0.10	88	29.6
0.15	135	30.0
0.25	230	30.1
0.50	430	30.4

Another experiment involved fermentation of 10 per cent dextrose solutions in the presence of varying amounts of sodium sulfite. The medium was a 10 per cent solution of dextrose in tap water containing 0.4 g. of steep liquor per 100 ml. of solution. The entire solution was inoculated from a Fleischmann yeast cake to give an initial count of 9 million cells per ml. The inoculated solution was distributed in 200 ml. portions between flasks which contained previously weighed quantities of sodium sulfite. Analysis was made by iodine titration after fermentation for 4 days. The results of the fermentations, as averages of the duplicates, are given in Table 7. The data show that some yeast proliferation occurred in the lower sulfite concentrations. However the maximum count reached was far lower than the range of 100 to 200 million cells per ml. normally found in alcoholic fermentations. The maximum yield of glycerol in this experiment occurred at 7 per cent sulfite concentration. A similar experiment using 5 per cent dextrose solutions gave the maximum yield of glycerol of 25.6 per cent on dextrose weight at 8 per cent sulfite concentration.

Since it was apparent that massive yeast inoculations gave better glycerol yields, a number of experiments were run in which various concentrations of dextrose were fermented in the presence of varying

amounts of sodium sulfite. The media were prepared by dissolving the required amount of sugar in tap water containing 0.4 g. steep liquor per 100 ml., and distributing 200 ml. portions between 300 ml. Erlenmeyer flasks containing previously weighed amounts of sodium sulfite. Each flask of medium was inoculated with one-fourth of a yeast cake (approximately 230 million cells per ml.). During the incubation period the flasks were shaken occasionally to suspend the yeast. Average results for the duplicate fermentations for several series are assembled in Table 8.

The maximum yield of glycerol occurred at a dextrose concentration of 10 per cent and sulfite concentration of 10 per cent. The highest amount of glycerol present was found in the flask containing 12.5 per cent dextrose and 15.6 per cent sodium sulfite, however. It is difficult to

TABLE 7

EFFECT OF SODIUM SULFITE CONCENTRATION UPON YEAST PROLIFERATION AND GLYCEROL YIELDS FROM DEXTROSE

Sodium Sulfite, g. per 100 ml.	Yeast Counts, Millions per ml.		Glycerol Yield, Per Cent on Dextrose
	Initial	Final	
1	9	25	6.5
5	9	20	22.1
6	9	16	23.3
7	9	16	24.1
8	9	12	22.4
9	9	9	16.0
10	9	7	4.0

determine from the data whether the ratio of sulfite to dextrose or the actual concentration of the sulfite is the more important factor in determining the yield of glycerol. Probably both factors are involved. The drop in yield of glycerol in higher concentration of sulfite might not have been experienced had the fermentation periods been prolonged. Analyses were made by iodine titration after fermentation for 3 days in the 5 and 10 per cent dextrose series, 4 days in the 7.5 per cent series, and 5 days in the 12.5 per cent series except where indicated in the table. It may be noted that the yields of glycerol for the same concentration of sulfite decreased with decreasing concentration of dextrose. In general it appeared that a 10 per cent dextrose solution containing 10 per cent sodium sulfite furnished the highest yield of glycerol.

A fermentation of dextrose in the presence of sodium sulfite was performed in larger volume with the *p*H recorded continuously by means of the Cameron *p*H Recorder. The medium consisted of 3 liters of solution containing 300 g. dextrose, 300 g. sodium sulfite and 6 ml. of

steep liquor in tap water. Two yeast cakes were used as inoculum. Samples were removed at intervals for glycerol analysis by iodine titration. The results are shown in Table 9.

The fermentation was apparently almost complete at 70 hours with a glycerol yield of 27.6% on dextrose. The *p*H did not rise and glycerol increased very slightly during the next 24 hours.

Since higher yields of glycerol were obtained with fermentation media containing high concentrations of sodium sulfite when heavy yeast inocula were used, it was of interest to investigate the effect of massive

TABLE 8

GLYCEROL PRODUCTION FROM MEDIA CONTAINING VARYING CONCENTRATIONS OF DEXTROSE AND SODIUM SULFITE USING LARGE INOCULA

Dextrose, g. per 100 ml.	Sulfite, g. per 100 ml.	Sulfite, Per Cent on Dextrose	Glycerol Yield, Per Cent on Dextrose
5	0.5	10	5.9
5	1.3	25	14.8
5	2.5	50	21.1
5	5.0	100	23.8
5	7.5	150	25.2
5	10.0	200	24.7
5	12.5	250	25.8
5	15.0	300	26.3
5	20.0*	400	28.8
7.5	1.9	25	15.1
7.5	3.8	50	23.7
7.5	5.6	75	25.8
7.5	7.5	100	26.9
7.5	9.4	125	27.9
7.5	11.3	150	28.2
7.5	20.0*	267	28.5
10	1.0	10	6.4
10	2.5	25	15.6
10	5.0	50	25.8
10	7.5	75	27.9
10	10.0	100	29.0
10	12.5	125	28.9
10	15.0	150	27.0
10	20.0	200	26.2
12.5	3.1	25	14.6
12.5	6.3	50	23.9
12.5	9.4	75	27.1
12.5	12.5	100	28.2
12.5	15.6	125	28.7
12.5	18.8	150	27.6

* Analyzed after fermentation for 8 days.

inoculation on the fermentation of dextrose in the presence of magnesium sulfite. Two fermentations were run. In one a solution of 30 g. dextrose in 600 ml. of tap water, with addition of one yeast cake and 30 g. magnesium sulfite was employed. In the other 70 g. dextrose in 740 ml. tap water, with one-third yeast cake, 1 g. yeast extract and 70 g. magnesium sulfite were used. The media were stirred continuously during fermentation by an air-driven stirrer. Data for the fermentations are given in Table 10. Glycerol was determined by iodine titration.

The first fermentation was complete in 20 hours with a yield of 19.9% of glycerol on dextrose. The second fermentation required 56 hours for completion, with a yield of 19.8 per cent of glycerol. The longer

TABLE 9

CHANGES IN GLYCEROL YIELD AND *p*H DURING FERMENTATION OF A DEXTROSE-SODIUM SULFITE MEDIUM

Time After Inoculation, Hrs.	*p*H	Glycerol Yield, Per Cent on Dextrose
1	9.0	1.5
4	8.5	2.0
22	7.0	12.7
35	6.9	19.5
46	7.0	24.1
58	7.0	26.8
70	7.0	27.6
94	7.0	27.7

time was to be expected since the inoculum was smaller and the dextrose concentration higher. The presence of yeast extract as nutrient did not affect the glycerol yield. Use of massive inoculation probably results in little or no yeast proliferation. The fermentations are more rapid but the yields of glycerol are not increased when dextrose is fermented in the presence of excess magnesium sulfite with massive yeast inoculation.

Evidently the glycerol yield obtained from dextrose fermentation in the presence of magnesium sulfite is dependent upon the concentration of sulfite present in solution as in the case of sodium sulfite fermentations. Since this is true, to obtain maximum glycerol yields the *p*H should be controlled at the most acid level possible without exceeding the bisulfite tolerance of the yeast. This limit seems to be about *p*H 6.0, and the most satisfactory range for control between *p*H 6.1 and 6.5. Under these conditions glycerol yields of about 45 per cent of theory are obtained and fermentations are essentially complete, the sugar not

converted to glycerol undergoing normal alcoholic fermentation. In the experiments with the insoluble sulfites reported above, the *p*H was either not controlled or was controlled roughly by intermittent addition of reagents as required at intervals of several hours. Two experiments were performed in which the *p*H was controlled by means of the Cameron *p*H Recorder-Controller by automatic addition of 50 per cent acetic acid as required. The medium for both of these fermentations

TABLE 10

FERMENTATION OF DEXTROSE IN THE PRESENCE OF MAGNESIUM SULFITE USING LARGE INOCULA

Initial Dextrose, Per Cent	Inoculum, Million Cells per ml.	Time of Fermentation, Hrs.	Glycerol Yield, Per Cent on Dextrose
5	300	20 (complete)	19.9
9.5	85	23	15.9
		56 (complete)	19.8

consisted of 1.5 liters of semi-synthetic medium containing 15 per cent dextrose and 280 g. magnesium sulfite hydrate. In the first fermentation the inoculum was 195 ml. of active yeast culture in semi-synthetic medium, in the second fermentation the inoculum was the cake of yeast and magnesium sulfite from the first fermentation. The results shown in Table 11 indicate that fairly good glycerol yields were obtained in both cases.

In order to facilitate recovery, if possible higher glycerol concentrations are desirable than are obtained by direct fermentation. Other

TABLE 11

FERMENTATION OF DEXTROSE IN THE PRESENCE OF MAGNESIUM SULFITE AT CONTROLLED *p*H

*p*H	Residual Dextrose, g. per 100 ml.	Glycerol Yield, Per Cent on Dextrose
6.5	0.124	23.4
6.2	0.070	23.2

investigators have reported the possibility of adding more sugar and refermenting the mashes to increase the glycerol content. It was found that refermentation was possible with both calcium sulfite and magnesium sulfite in which liquid from one fermentation was used in preparing media for a second. For example, a sample of 200 ml. of the fermentation beer from the first magnesium sulfite fermentation of Table 11 was made alkaline with magnesium hydroxide, and volatile materials were removed by distillation of one-third of the volume. The

residue was filtered, 30 g. of dextrose added, and the mixture diluted to 200 ml. This was inoculated with 30 ml. of active yeast culture in semi-synthetic medium, and a sample of this inoculated medium analyzed for sugar and glycerol. To the flask were added 40 g. of magnesium sulfite hydrate, and the fermentation incubated for 3 days. The *p*H was not controlled, but was 6.3–6.4 during the most active fermentation period. The initial glucose content was 13.04 g. and glycerol content 2.69 g. per 100 ml. The final glycerol concentration after the fermentation was 5.05 g. per 100 ml. Thus the increase in glycerol content due to the second fermentation was 2.36 g. per 100 ml., which corresponds to a yield of 18.1 per cent on the weight of dextrose.

DISCUSSION AND CONCLUSIONS

The results of the experimental work on possible production of glycerol by fermentation of dextrose showed that use of ammonium compounds was not practical. Both of the insoluble sulfites tried, calcium sulfite and magnesium sulfite, resulted in increased yields of glycerol from dextrose fermentation. However, glycerol yields under the best conditions found were about twice as good with magnesium sulfite as with calcium sulfite. Studies with sodium sulfite fermentations indicated that the glycerol yields obtainable are a function of the sulfite concentration present in solution, and with the slightly soluble sulfites this is, of course, a function of the solubility. Since magnesium sulfite is more soluble than calcium sulfite this accounts for the increased glycerol yields with the former. These yields correspond closely with the yields obtained with sodium sulfite in equivalent concentration.

Experiments with sodium sulfite and magnesium sulfite indicated that more rapid fermentations were secured by use of relatively massive inoculations of yeast since proliferation of yeast is inhibited by sulfite. For high sulfite concentrations massive inoculations are mandatory if fermentation is to occur.

Glycerol yields were most satisfactory for fermentations with calcium and magnesium sulfites when the *p*H levels of the media were kept on the acid side of neutrality, but sufficiently high that the concentration of bisulfite did not become inhibitory to the yeast. Control of the *p*H of the fermentations in these experiments was by means of ordinary acids such as sulfuric and acetic acids. Use of these acids would not be advisable in actual practice for the production of glycerol since they would result in formation of soluble salts which could not readily be removed from the fermentation beers. Most suitable reagent for adjusting and controlling the *p*H would be sulfur dioxide, which would give sulfurous acid in solution, and not complicate the situation by adding anions not already present. Equipment was not available during the course of this work for control by means of sulfur dioxide. Hickey (10) later found that glycerol yields were increased some 10 per cent by initially adjusting the *p*H to about 6.1 by addition of sulfur dioxide after

the magnesium sulfite had been added. He also found that repeated adjustment by means of sulfur dioxide to this *p*H level during fermentation further increased the glycerol yield slightly.

Although the highest yields obtained in the reported experiments were in the neighborhood of 23–24 g. of glycerol per 100 g. of dextrose, representing approximately 46 per cent of theory yield, the sugar dissimilations were essentially complete, and the sugar not converted to glycerol underwent the normal alcoholic fermentation. Undoubtedly the yields could be somewhat improved by adjustment of *p*H by means of sulfur dioxide before and during the fermentation, but yields of more than 50 per cent of theory would not be regularly anticipated. Even with yields at this level, the process would have practical importance if operated in an alcohol plant. The by-products would be ethanol and acetaldehyde, and with this process the recovery of glycerol should be greatly facilitated, since soluble compounds would be minimized.

The process would involve fermentation of the sugar in the presence of excess magnesium sulfite by acclimatized yeast, which could be efficiently re-used, with agitation, and control of *p*H at 6.0–6.5 by means of sulfur dioxide additions as required. Upon completion of the fermentation, the beer would be rendered alkaline by means of a slurry of magnesium hydroxide, and acetaldehyde and ethanol recovered by distillation. The insoluble sulfite remaining would be removed by filtration, yielding a filtrate containing glycerol and relatively small amounts of dissolved solids. After evaporating the solution the glycerol would then be recovered by conventional methods such as steam or vacuum distillation, or solvent extraction. A patent on the fermentation process has been issued to Fulmer, Underkofler and Hickey (7) and assigned to the Iowa State College Research Foundation.

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PRODUCTION OF GLYCEROL BY FERMENTATION

II. Fermentation of Disaccharides and Starch

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Received July 26, 1951

Fermentation processes for production of glycerol have not gained industrial acceptance mainly because of the difficulty in recovering the glycerol from the fermented beers. This difficulty has been due to the necessity of using large quantities of soluble alkalis or soluble sulfites in the fermentations. Conventional methods of distillation or solvent extraction have not been found satisfactory for removing glycerol from the resulting beers containing a little glycerol and much extraneous soluble material.

Research leading to a successful method for producing glycerol by yeast fermentation of dextrose in the presence of magnesium sulfite was described in the first paper of this series (6). This procedure has an advantage over those previously suggested in that the amount of soluble salts left in the fermented beers is low. Highest yields of glycerol were obtained from the fermentation of dextrose in the presence of excess magnesium sulfite when the *p*H was controlled between 6.0 and 6.5. Under optimum conditions by this fermentation procedure at least 20 per cent yields of glycerol, based on sugar weight (46 per cent of theory), are obtainable. The balance of the sugar not converted to glycerol undergoes normal alcoholic fermentation. The process therefore yields as major products glycerol, acetaldehyde and ethanol. Such a process if operated at all would probably be in connection with an industrial alcohol plant.

Should it be desired to produce glycerol by fermentation on the industrial scale, a cheaper substrate than dextrose would be necessary in order to be competitive. The raw materials for yeast fermentation to produce ethanol have long been molasses and starchy materials such as grains and potatoes. These are the cheapest and most abundant materials for fermentation industries.

Although Eoff (1) based an alkaline fermentation procedure for glycerol production on the use of blackstrap molasses, recovery of glycerol in his large scale tests, reported by Eoff, Lindner and Beyer (2), were only about 50 per cent of the glycerol present. Use of blackstrap molasses by any of the proposed glycerol fermentation processes adds to the complications of recovery because of the high concentration of

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unfermentable solids present which may be as high as 35 per cent of the molasses' weight. Molasses mashes containing 10 per cent of sugar would then have approximately 7 g. of unfermentable solids per 100 ml.

High test molasses would be a much more favorable substrate. The unfermentable solids content of high test molasses is usually less than 10 per cent, and a mash made from this substrate containing 10 per cent sugar would have only about 1 g. of unfermentable solids per 100 ml.

With the expectation of fermenting high test molasses an industrial concern was licensed under the patent of Fulmer, Underkofler and Hickey (3) early in the period of World War II. Unfortunately at this time high test molasses, and in fact blackstrap molasses, became unavailable due to the war conditions. This industrial concern therefore investigated the possibility of using the magnesium sulfite glycerol fermentation process for the fermentation of clear filtrates from malt-saccharified grain mashes. The results were very disappointing; the fermentations were slow and sluggish, very incomplete, and the glycerol yields extremely poor. These discouraging results led to the conclusion that processing grain mashes was impractical, and the license agreement was cancelled. The unsatisfactory results with malt-saccharified mashes led to an extensive investigation, here reported, on production of glycerol from crude sugar mixtures, disaccharides and starch by the magnesium sulfite fermentation process.

MATERIALS AND METHODS

Substances employed as fermentation substrates included several sugars and starch of pure or technical grades, and crude carbohydrate mixtures. The substrates used in the various series will be described as the experiments are discussed. Unless otherwise indicated steep liquor concentrate, obtained from the American Maize-Products Co., was the only nutrient employed in the mashes. In general, unless otherwise stated, the mashes were not sterilized before inoculation and fermentation since experience had shown that the presence of sulfites effectively inhibited bacterial contamination. Commercial magnesium sulfite hexahydrate ($MgSO_3 \cdot 6H_2O$) was employed, and fermentations were incubated at 30°C. unless otherwise indicated. Massive inoculations employing a slurry of Fleishmann yeast cake in water were generally employed. For some experiments, as indicated, laboratory yeast cultures were grown for inoculum.

The yeast culture used previously, *Saccharonyces cerevisiae* No. 43, was employed unless otherwise stated, and laboratory yeast cultures were grown on a sterilized medium containing 10 g. dextrose and 0.6 ml. steep liquor concentrate per 100 ml.

Glycerol analyses were made by the iodine titration method described in the previous paper (6). Where ethanol was determined, this was done by distilling an aliquot of the fermented beer into a volumetric flask, and measuring the specific gravity (25°/25°) of the distillates.

Sugar analyses were made by the method of Underkofler, Guymon, Rayman and Fulmer (7).

EXPERIMENTAL

Fermentation of Crude Sugar Mixtures

To ascertain whether mixtures of sugars from natural sources could be satisfactorily fermented in the presence of magnesium sulfite in the same manner as was successful with dextrose, blackstrap molasses and high test molasses mashes were subjected to fermentation. Duplicate flasks containing media prepared from Louisiana blackstrap molasses and Cuban high test molasses were prepared. Each 500–ml. Erlenmeyer flask contained 300 ml. of medium obtained by dilution of the molasses with tap water to give a sugar concentration of about 10 per cent. A sample of each medium was analyzed for sugar content, then 60 g. of magnesium sulfite hydrate was added to each flask, the *p*H adjusted to 6.5 by adding sulfuric acid and each medium inoculated with one-fourth of a yeast cake. After incubation for 4 days, glycerol analyses were made.

The blackstrap molasses medium, with an initial sugar content of 10.2 g. per 100 ml., gave a glycerol yield of 19.9 per cent on sugar present while the high test molasses, with an initial sugar content of 10.4 g. per 100 ml., gave a glycerol yield of 21.1 per cent. Fermentations were satisfactory and the glycerol yields were of the same order as had previously been obtained from dextrose fermentations. High test molasses gave slightly better glycerol yields than Louisiana blackstrap molasses.

Several preliminary fermentations were run using filtrates from malted corn mashes to which magnesium sulfite was added. Yields of glycerol of more than 10 per cent of the sugar weight were rarely obtained, and the fermentations were sluggish and prolonged. The highest glycerol yield obtained in any of these experiments was with a filtrate obtained by malting a cooked corn mash for 30 hours at 55°C. Two 250–ml. portions of this filtrate, which analyzed 9.44 g. maltose hydrate per 100 ml., were fermented after addition of 20 g. magnesium sulfite and inoculation with 20 ml. of active laboratory yeast culture. The average yield of glycerol after incubation for 7 days was 15.4 per cent the sugar weight.

A series of experiments was run in which various starchy materials were saccharified by means of malt or mold bran (*Aspergillus oryzae*). The materials employed included corn starch, yellow corn meal and white corn meal. The procedure for preparing the mashes was as follows: 30 g. of the starchy material were mixed with 300 ml. of 0.04 *N* hydrochloric acid, gelatinized by heating, and cooked for one hour at 15 lbs. steam pressure. To the hot mash about 1 g. of calcium carbonate was added, and after the mash had cooled to the proper temperature 1.8 g. of the amylolytic material were added. For the malt saccharifications the mash was held at 55°C. for 5 hours; for the mold bran the mash was held at 30°C. for 2 hours. After the saccharification period

25 g. magnesium sulfite were added and 30 ml. of active laboratory yeast culture. During incubation the flasks were shaken at intervals, and all were analyzed at the end of 5 days. The average values for duplicate fermentations in a typical series are shown in Table 1. Glycerol yields were in the range of 12 per cent, and no great differences were apparent in the mashes saccharified by mold bran or by malt. Yeast strain No. 53 (*Saccharomyces ellipsoideus* var. Steinberg) seemed to give somewhat better yields than the regular laboratory culture No. 43.

TABLE 1

FERMENTATION OF ENZYME CONVERTED STARCHY MATERIALS IN THE PRESENCE OF MAGNESIUM SULFITE

Starchy Material	Enzyme Source	Yeast Strain, No.	Glycerol Yield, Per Cent of Starch
Corn starch	Mold bran	43	13.2
Yellow corn meal	Mold bran	43	10.5
Yellow corn meal	Malt	43	11.2
White corn meal	Mold bran	43	11.5
White corn meal	Mold bran	53	12.9
White corn meal	Malt	53	12.6

The reason for the poor utilization of the maltose in the enzyme-saccharified mashes might be ascribed to the specific effect of the magnesium or sulfite ions or to the salt concentration. In order to examine this effect a corn mash was prepared, saccharified by malt, and filtered. Into each of four flasks 300-ml. portions of the filtrate were placed, and each inoculated with 30 ml. of active yeast culture. Two hours after inoculation, to separate flasks were added 10 g. magnesium sulfite, 10 g. magnesium sulfite and 1 g. secondary potassium phosphate trihydrate, 9 g. magnesium sulfate, or 11 g. sodium sulfate, respectively. These amounts made the concentrations of the salts about 0.25 *M*, approximately the concentration in which magnesium sulfite is soluble when used in a fermentation. The sugar utilization was followed by polarimetry. At the end of 3 days about 75 per cent of the maltose had been fermented in the media containing the sulfates, but in those containing magnesium sulfite only 15 per cent of the maltose had been utilized.

The definite inhibiting effect of magnesium sulfite upon the fermentations in this experiment leads to the conclusion that sulfite is the cause of the poor fermentations of maltose in enzymatic starch hydrolyzates. Since yeast ferments dextrose satisfactorily in the presence of magnesium sulfite, it is apparent that in some way sulfite hinders the fermentation of maltose.

In contrast with the poor fermentality of enzyme-saccharified corn mashes, acid hydrolyzed starch was found to ferment satisfactorily. A

sample of corn starch was hydrolyzed by autoclaving 22.5 g. of starch with 300 ml. of 0.02 *N* sulfuric acid at 25 lbs. steam pressure for 4 hours. The reducing sugar analysis, as dextrose, was 6.21 g. per 100 ml., corresponding to 84.5 per cent conversion. To the solution 25 g. magnesium sulfite and 1 ml. of steep liquor were added and the *p*H adjusted to 6.0. The medium was inoculated with 20 ml. of active culture of yeast and incubated. After 6 days the glycerol yield was found to be 22.1 per cent of the reducing sugar weight or 25 per cent on original dry starch.

Fermentation of Pure Sugars

Since the fermentation of maltose in the presence of sulfite in enzyme-saccharified starch mashes was unsatisfactory, some experiments were run on the fermentation of several sugars. In one series 20 g. each of maltose hydrate, lactose hydrate, sucrose and dextrose were weighed into 300–ml. Erlenmeyer flasks in duplicate. To each flask 200 ml. of tap water and 1 ml. of steep liquor were added, and the media sterilized at 15 lbs. steam pressure for 15 minutes. After cooling, to each flask were added 25 g. of magnesium sulfite and 20 ml. of 42-hour culture of yeast No. 43. After 5 days incubation the dextrose and sucrose media had almost ceased to show evidence of gassing, while the lactose and maltose media still had shown no sign of activity. The average glycerol yields, based on sugar weight were 1.5, 1.6, 18.2 and 18.4 per cent, respectively, for the lactose, maltose, sucrose and dextrose. Lactose is known not to be fermented by many yeast strains so the low yield of glycerol from this sugar is understandable. However, the poor fermentation of maltose is still without explanation. Sucrose and dextrose fermented satisfactorily.

The effect of massive inoculation upon sulfite fermentation of several sugars was investigated using small tube fermentations. Into separate test tubes were placed 1 g. sodium sulfite, one drop of steep liquor concentrate, 1 g. sugar and 10 ml. of water. Each tube was then inoculated with a small piece of yeast cake which represented a very large inoculum. After 4 days incubation the contents of the tubes were analyzed. The glycerol yields, based on sugar weight, were 0.2, 1.0, 30.0 and 29.0 per cent, respectively, for trehalose, maltose, sucrose and dextrose. The yields of glycerol from both sucrose and dextrose were excellent, while the fermentation of the other two sugars, trehalose and maltose, was negligible. Failure of maltose to ferment in the presence of sodium sulfite was the same as in the presence of magnesium sulfite.

Four strains of yeast were tested as to their effectiveness in glycerol production by fermentation of dextrose and maltose. Three strains of *S. cerevisiae,* designated as Nos. 43, 16 and 51, and a strain of *S. ellipsoideus,* var. Steinberg, designated as No. 53, were employed. These cultures were carried through three successive 36-hour subcultures in 5 per cent dextrose-steep liquor medium starting from stock slants on wort agar. The third transfer was used as inoculum in each case. For the fermentations duplicate flasks of media containing 10 per cent of each sugar with 0.4 per cent steep liquor and 20 g. magnesium sulfite

in 200–ml. portions were used. To duplicate flasks of each sugar medium were added 20 ml. of the yeast culture. The flasks were shaken periodically during incubation and analyzed after 5 days. The results are shown in Table 2. From the data it is apparent that regular laboratory yeast No. 43 gave the highest yield of glycerol from dextrose. However, yeast No. 43 was the poorest for maltose and the strain of *S. ellipsoideus*, No. 53, was the best of the four strains for fermentation of maltose. The yields of glycerol obtained from maltose were noticeably less than those obtained from dextrose with all of the yeast strains.

TABLE 2

FERMENTATION OF DEXTROSE AND MALTOSE IN THE PRESENCE OF MAGNESIUM SULFITE BY FOUR YEAST CULTURES

Sugar	Yeast No.	Glycerol Yield, Per Cent on Sugar
Dextrose	16	16.4
Dextrose	43	19.6
Dextrose	51	18.7
Dextrose	53	17.9
Maltose	16	12.7
Maltose	43	5.2
Maltose	51	12.2
Maltose	53	13.8

The fermentation of maltose in the presence of sodium sulfite was investigated in some detail in order to attempt to ascertain why glycerol yields from this sugar were so much poorer than from dextrose or sucrose. Using both technical grade of maltose and the C. P. sugar, experiments were run repeatedly to see if addition of nutrients, varying the strain of yeast or altering the physical conditions of the medium would increase the yields of glycerol. Results were inconclusive, and frequently did not check when experiments were repeated. It was very apparent that under all conditions tried maltose fermented poorly in the presence of sodium sulfite, and maximum yields of glycerol were about 12 per cent of the sugar weight after 4-day fermentations.

Addition of various nutrients showed that maltose fermentation was not aided significantly by addition of malt, mold bran, yeast extract, alfalfa, steep liquor, phosphates or ammonium salts. Addition of dextrose caused rapid fermentation to occur. But 4-day analyses showed that while the dextrose had been fermented completely, most of the maltose remained unfermented. Variation of the *p*H showed that the optimum reaction was between *p*H 6.9 and 7.1 for fermentation of maltose in the presence of sodium sulfite.

Although in all of the 4 or 5 day fermentations tried the maximum glycerol yields obtained in fermentation of maltose in the presence of sulfites was about 12 per cent of the sugar weight, it was noted that in many cases feeble gassing seemed still to be taking place in these fermentations at the time of analysis. It was considered possible that the rate of fermentation was slow and that fermentation for longer periods might result in better glycerol yields. Two prolonged fermentations were therefore run in which the *p*H was continuously recorded by a Cameron *p*H Recorder. In one experiment the *p*H was not controlled, in the other the *p*H was controlled at *p*H 7.0 ± 0.1 by automatic addition of hydrochloric acid or sodium hydroxide as required. The media were prepared by dissolving 150 g. maltose, 120 g. sodium sulfite and 6 ml. sterile steep liquor in tap water and bringing the total volume to 2,700 ml. Each flask of medium was inoculated with 300 ml. of 48-hour culture of yeast No. 53 in 5 per cent maltose-steep liquor medium, and the *p*H adjusted to 7.1. Samples were removed at intervals during incubation and analyzed for glycerol yields. The data are presented in Table 3.

TABLE 3

PROLONGED FERMENTATION OF MALTOSE IN THE PRESENCE OF SODIUM SULFITE

Uncontrolled *p*H			Controlled *p*H		
Age in hrs.	*p*H	Glycerol Yield, Per Cent of Sugar	Age in hrs.	*p*H	Glycerol Yield, Per Cent of Sugar
5	7.1	0.5	17	7.1	1.3
23	7.0	1.4	41	6.9	4.9
46	6.8	5.1	65	6.9	8.6
70	6.7	7.5	90	7.0	10.1
94	6.7	9.7	113	7.1	12.2
118	6.7	11.1	137	7.1	12.9
142	6.7	12.8	161	7.0	14.2
166	6.7	14.2	185	7.0	15.1
190	6.7	15.5	209	7.0	15.7
214	6.8	16.4	233	7.0	16.6
238	6.9	17.9	281	7.0	17.8
286	7.0	19.2	329	7.0	18.3
310	7.3	19.9	377	7.0	18.5
338	7.3	20.4	.....	.....	.....
362	7.3	20.8	.....	.....	.....

In both fermentations the glycerol yields seemed to increase continuously up to the time they were discontinued, at 15 days. At this time the rate of increase had become so small that the analytical error was greater than the amount of increase. In the case of the uncontrolled fermentation the final glycerol yield amounted to 20.8 per cent of initial maltose and for the controlled fermentation the yield was 18.5 per cent. The final yields of glycerol obtained in these prolonged fermentations were of the same order as the yields obtained from dextrose fermented for 4 days under similar conditions. That is, dextrose was fermented about four times as fast as maltose, and the prolonged fermentation periods necessary for the maltose would be quite impractical for industrial use.

Fermentation of Starch

The sluggish fermentation of maltose in the presence of sulfites rules out the possibility of employing enzyme converted starchy mashes for the glycerol fermentation. However, acid hydrolysis of starch, resulting in dextrose production, might prove more feasible. Results reported above showed that acid hydrolyzed starchy materials fermented satisfactorily in the presence of sulfite.

As starchy raw materials, whole grains would not be suitable due to the large amount of residual solids which would render recovery of glycerol impractical. Separated starch from the wet-milling industry should prove suitable, as might also by-product starch slurries such as are produced in the separation of gluten from wheat flour. The latter process is used as one industrial source for glutamic acid and monosodium glutamate. The residual starch slurry can be most economically utilized by means of fermentation. Both of these sources of starch were investigated as possible substrates for glycerol fermentation.

The hydrolysis of starch by acid is an accepted commercial process. The factors of acid concentration, temperature and time are interrelated variables. Conditions may be chosen for maximum conversion of starch to sugar. Industrially, for the production of dextrose sugar from corn starch, approximately 18 per cent starch slurries are acidified with hydrochloric acid to about *p*H 1.5 and converted at 40 to 45 lbs. steam pressure for about 30 minutes. Ruf, Stark, Smith and Allen (5) developed an acid hydrolysis process which is satisfactory for industrial fermentation purposes. Based upon the available equipment, for our laboratory work the following conditions were chosen: 0.1 *N* sulfuric acid at 25 lbs. steam pressure for 2 hours. Under these conditions conversion was 98 to 100 per cent complete as based upon reducing sugar analyses.

The general procedure was as follows: Quantities of 30 g. starch, unless otherwise stated, were weighed into separate 500-ml. Erlenmeyer flasks. To each flask were added 300 ml. of 0.1 *N* sulfuric acid. The starch was gelatinized by heating the mixtures in a boiling water bath, with frequent shaking, then the flasks were placed in an autoclave and heated for 2 hours at a steam pressure of 25 lbs. In some cases the acid was

neutralized by addition of the calculated quantity of solid calcium carbonate, in other cases sulfites to be employed were added and the *p*H adjusted to the desired value by addition of sodium hydroxide or hydrochloric acid.

For all fermentations of acid hydrolyzed starch mashes, massive inoculation from Fleishmann yeast cakes was employed. Massive inoculation and re-use of yeast have been found practical and desirable in industrial alcoholic fermentations of waste sulfite liquor, wood hydrolyzates and molasses, and are common industrial practice for the first two substrates named.

Preliminary fermentations were run using corn starch hydrolyzed as above described, with 30– and 60–g. portions of the sulfites of magnesium, calcium and ammonium added to separate flasks in duplicate, and *p*H adjusted to 6.0 after the addition of sulfite. Glycerol yields were practically identical with either quantity of the salts employed, and much better with the magnesium sulfite than the others, the average glycerol yield in per cent of dextrose equivalent being 23.2, 4.3 and 1.4, respectively, for the magnesium, calcium and ammonium sulfites. In another experiment freshly precipitated magnesium sulfite hexahydrate was compared with a commercial sample, and both gave identical results within the limits of experimental error.

Various additions of calcium, ammonium and sodium sulfites to fermentations in the presence of magnesium sulfite showed no advantage of using mixed sulfites. Glycerol yields were in no case improved materially over fermentation with magnesium sulfite alone, and frequently were depressed.

For a commercial process both the sulfite and yeast would probably be recovered and used over in subsequent fermentations. Acid hydrolyzed starch media were prepared, neutralized by means of calcium carbonate to *p*H 6.3, magnesium sulfite added and each flask inoculated with one-third of a yeast cake. The fermentations were incubated for 65 hours, analyzed, and the media filtered. The cakes of sulfite and yeast were added to a duplicate set of hydrolyzed starch media. To one pair of duplicate flasks no further additions of sulfite or yeast were made. To other pairs various amounts of fresh sulfite or yeast or both were added as indicated in Table 4. The second fermentations were analyzed after 65 hours, and the results are shown in Table 4.

Both the first and second fermentations gave reasonably good glycerol yields. Addition of more yeast resulted in better fermentations than when no more was added. This was undoubtedly due partly to drying during filtration. However, little or no multiplication of yeast occurs during the fermentations, and addition of some fresh yeast to each fermentation batch would be desirable for commercial practice.

Previous work (6) had shown that a slightly acid medium was desirable for fermentations of dextrose in the presence of magnesium sulfite or calcium sulfite. For magnesium sulfite the optimum *p*H was about 6.0 to 6.5. Acid hydrolyzed starch medium was prepared in the

TABLE 4

RE–USE OF YEAST AND MAGNESIUM SULFITE IN FERMENTATION OF ACID HYDROLYZED STARCH

First Fermentation		Second Fermentation		
Sulfite Added, g.	Glycerol Yield, Per Cent on Sugar	Sulfite Added, g.	Yeast Added, Cakes	Glycerol Yield, Per Cent on Sugar
60	20.8	0	0	15.1
60	20.7	0	⅓	20.1
30	20.2	30	0	17.1
30	19.9	30	⅓	19.7
30	20.4	15	0	16.8
30	20.0	15	⅓	19.0

usual manner. After hydrolysis 30 g. magnesium sulfite were added to each flask and duplicate flasks of medium adjusted to the *p*H levels shown in Table 5 by addition of required amounts of concentrated sodium hydroxide or hydrochloric acid. Each flask of medium was then inoculated with one-third of a yeast cake and incubated. At 20-hour intervals the *p*H was measured and readjusted where necessary. After 60 hours the fermentations were analyzed for glycerol and ethanol production with the results shown in Table 5. The data show that the fermentations were influenced quite markedly by the *p*H of the medium used. The optimum seemed to be in the range *p*H 6.0–6.5, which agrees with that previously found for fermentation of dextrose. At the *p*H level of 6.0, the yield of glycerol was 44.1 per cent of theory and of ethanol 36.3 per cent of theory, together representing 80.4 per cent of theory conversion of the dextrose equivalent present. This is about as satisfactory as fer-

TABLE 5

FERMENTATION OF ACID HYDROLYZED STARCH IN THE PRESENCE OF MAGNESIUM SULFITE AT VARIOUS *p*H LEVELS

*p*H			Glycerol Yield, Per Cent on Sugar	Ethanol Yield, Per Cent on Sugar
Initial	At 20 hrs.	At 40 hrs.		
5.0	5.2	5.0	13.9	0.3
5.5	5.5	5.5	9.8	0.9
6.0	6.1	6.0	22.5	18.5
6.5	6.4	6.5	22.7	16.5
7.0	6.8	6.9	19.1	22.2
7.5	7.0	7.1	17.5	20.5

mentations of acid hydrolyzed starch by the normal alcoholic fermentation without addition of fungal amylase (5).

For commercial purposes it is desirable to use mash concentrations as high as will give good yields of fermentation product. Likewise a certain latitude in fermentation temperature is desirable. Experiments were therefore run using varying starch concentrations at two temperatures. The mashes were hydrolyzed as usual with 300 ml. of 0.1 *N* sulfuric acid cooked at 25 lbs. steam pressure for 2 hours using different weights of starch in different flasks. After cooling, magnesium sulfite equal to the weight of starch taken for each flask was added, and each inoculated with one-third of a yeast cake. Results of the hydrolyses and fermentations are given in Table 6. It is apparent from the data that yields of glycerol were poorer with increased starch concentrations.

TABLE 6

FERMENTATION OF DIFFERENT CONCENTRATIONS OF STARCH HYDROLYZATES IN THE PRESENCE OF MAGNESIUM SULFITE AT TWO TEMPERATURES

Concentration of Starch, Per Cent	Conversion of Starch, Per Cent as Dextrose	Temperature, °C.	Glycerol Yield, Per Cent on Dextrose
5	100.2	30	22.7
10	95.7	30	22.0
20	93.6	30	17.0
5	100.2	37	22.5
10	95.7	37	21.9
20	93.6	37	17.2

Conversion to sugar was also lower at the higher starch concentrations. Yields were satisfactory, however, at 10 per cent starch concentration.

Various other expedients were investigated in an effort to improve glycerol yields from cornstarch mashes. These included addition of magnesium sulfite to fermentations of starch hydrolyzates after the fermentations had become active rather than at the start of fermentation, acclimatization of the yeast cake by incubation in sugar solution in the presence of magnesium sulfite, and activation of the yeast by incubation with sugar and nutrients before using as inoculum. None of these procedures affected the glycerol yields obtained.

Procedures found most favorable for glycerol fermentation of acid hydrolyzed cornstarch mashes were applied to the fermentation of starch slurries obtained after removal of gluten from wheat flour. The procedure for obtaining the starch slurry was as follows: 1,000 g. of flour "clears" were mixed with 1,000 ml. of tap water at 52°C. The mixture was allowed to stand for 30 minutes and then 2,000 ml. of tap water at 52°C. mixed in carefully. The gluten was separated on a vibrating

screen and the starch slurry collected for use. Analyses showed the slurries so obtained in various batches contained 21 to 24 g. starch per 100 ml. To prepare fermentation media starch slurry was mixed with an equal volume of sulfuric acid solution and cooked for 4 hours at 15 lbs. steam pressure. Variation of acid concentration showed that most satisfactory conversion resulted, with the time and temperature employed, when 0.3 *N* sulfuric acid was used, which represents a final acid normality of 0.15 in the cooks. Fermentations were run in 300–ml. quantities in 500 ml.-Erlenmeyer flasks, to each of which was added sulfite and one-third yeast cake for inoculum. Various sulfites were employed, and dextrose solu-

TABLE 7
FERMENTATION OF ACID HYDROLYZED WHEAT STARCH SLURRY

Initial Reducing Sugar, g. per 100 ml.	Sulfite, g.	*p*H	Glycerol Yield		Ethanol Yield		Total Products, Per Cent of Theory	
			Per Cent on Sugar	Per Cent on Starch	Per Cent on Sugar	Per Cent on Starch	on Sugar	on Starch
10.25	20 $MgSO_3$	5.5	15.6	16.7	.....	.....	.....	.....
10.25	20 $MgSO_3$	6.0	11.1	11.9	.....	.....	.....	.....
10.25	20 $MgSO_3$	6.5	27.2	29.2	17.5	18.8	87.4	84.2
10.43	20 $MgSO_3$	6.5	27.1	29.1	15.9	17.1	84.1	80.6
10.25	20 $MgSO_3$	7.0	24.2	26.0	19.7	21.2	85.9	82.8
10.43	20 $MgSO_3$	7.0	24.4	26.2	15.8	17.0	78.5	75.1
10.43	13 Na_2SO_3	7.3	3.8	.....	.....	.....	.....	.....

tions were fermented as controls. Of the sulfites, calcium sulfite and ammonium sulfite gave poor fermentations while magnesium sulfite and sodium sulfite gave results practically equivalent to those previously obtained for these salts with acid hydrolyzed cornstarch or dextrose fermentations. The optimum *p*H was found to be about 6.5. Representative results are given in Table 7, the values given being the averages for duplicate fermentations. In the fermentations at *p*H 6.5 the average glycerol yield was 27.1 per cent on reducing sugar present (53.1 per cent of theory) and 29.1 per cent on original starch (51 per cent of theory). Ethanol yields brought the percentage conversion in these fermentations to 84.1–87.4 per cent of theory on reducing sugar present and 80.6–84.2 per cent on starch.

DISCUSSION

The fermentation of various substrates in the presence of excess amounts of magnesium sulfite for glycerol production has been found to be generally practical. The method was originally developed for dex-

trose in a semisynthetic medium (6). The optimum conditions for maximum glycerol yields from dextrose were *p*H of 6.0 to 6.5, preferably controlled at this level, excess magnesium sulfite and massive yeast inoculation. Other substrates which have been successfully fermented with optima being found the same as for dextrose, are blackstrap molasses, high test molasses, sucrose, and acid hydrolyzed cornstarch or wheat starch. Enzyme converted grain mashes are not satisfactory substrates for sulfite fermentations. This is due to poor fermentability of maltose in the presence of sulfites. Fermentation of maltose in the presence of sulfites is very sluggish and prolonged, and no means of improving the fermentation of this sugar has been found.

Recent industrialization of the synthetic process for glycerol manufacture makes rather unpromising the production of glycerol by fermentation at the present time. However, the economics of the magnesium sulfite process would make it successful at the present price levels for glycerol, acetaldehyde and ethanol. The most favorable substrates would be high test molasses or acid hydrolyzed starch slurries obtained as by-products from other operations.

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